

# Mesenchymal Stem Cells: Immunology and Therapeutic Benefits

Najib El Haddad  
Transplantation Research Center,  
Brigham & Women's Hospital, Harvard Medical School  
USA

## 1. Introduction

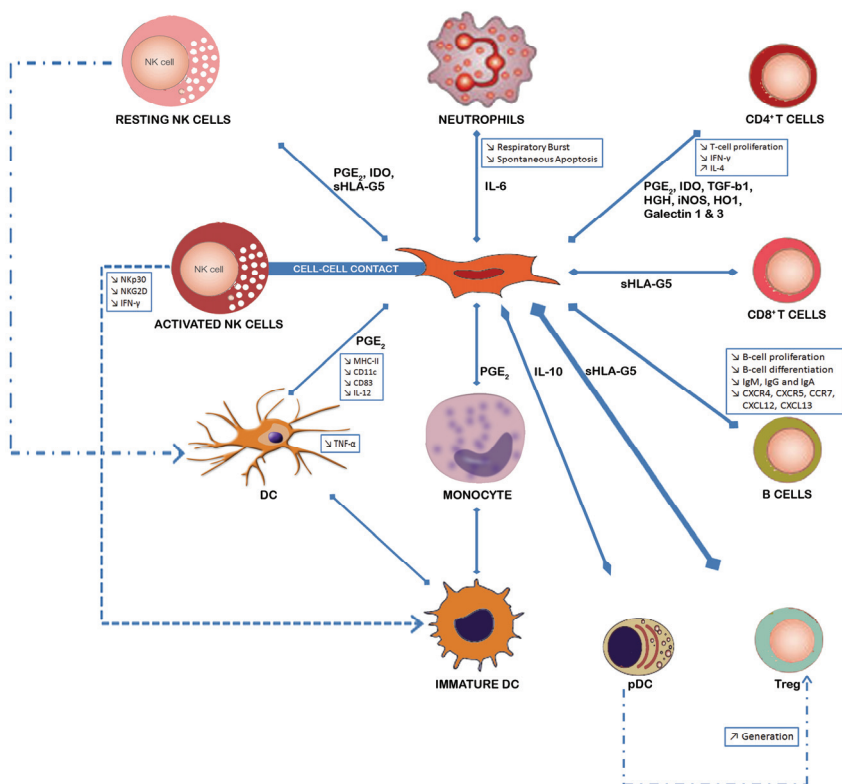
Bone marrow is a complex tissue containing hematopoietic cell progenitors and their progeny integrated within a connective-tissue network of mesenchymal-derived cells known as the stroma (1). The stroma cells, or Mesenchymal stem cells (MSCs), are multi-potent progenitor cells that constitute a minute proportion of the bone marrow, represented as a rare population of cells that makes up 0.001 to 0.01% of the total nucleated cells. They represent 10-fold less abundance than the haematopoietic stem cells (2), which contributes to the organization of the microenvironment supporting the differentiation of hematopoietic cells (3). MSC are present in tissues of young, as well as, adult individuals (4, 5), including the adipose tissue, umbilical cord blood, amniotic fluid and even peripheral blood (1, 6-8). MSCs were characterized over thirty years ago as fibroblast-like cells with adhesive properties in culture (9, 10). The term MSC has become the predominant term in the literature since the early 90s (11), after which their research field has grown rapidly due to the promising therapeutic potential, resulting in an increased frequency of clinical trials in the new millennium at an explosive rate.

As data accumulated, there was a need to establish a consensus on the proper definition of the MSCs. The International Society for Cellular Therapy has recommended the minimum criteria for defining multi-potent human MSCs (12, 13). The criteria included: (i) cells being adherent to plastic under standard culture conditions; (ii) MSC being positive for the expression of CD105, CD73 and CD90 and negative for expression of the haematopoietic cell surface markers CD34, CD45, CD11a, CD19 or CD79a, CD14 or CD11b and histocompatibility locus antigen (HLA)-DR; (iii) under a specific stimulus, MSC differentiate into osteocytes, adipocytes and chondrocytes *in vitro*. These criteria presented properties to purify MSC and to enable their expansion by several-fold *in-vitro*, without losing their differentiation capacity. When plated at low density, MSCs form small colonies, called colony-forming units of fibroblasts (CFU-f), and which correspond to the progenitors that can differentiate into one of the mesenchymal cell lineages (14, 15). It has been reported lately that MSCs are able to differentiate into both mesenchymal, as well as, non-mesenchymal cell lineages, such as adipocytes, osteoblasts, chondrocytes, tenocytes, skeletal myocytes, neurones and cells of the visceral mesoderm, both *in vitro* and *in vivo* (16, 17).

All cells have half-lives and their natural expiration must be matched by their replacement; MSCs, by proliferating and differentiating, can be the proposed source of these new replacement cells as characterized in their differentiation capacity. This replacement hypothesis mimics the known sequence of events involved in the turnover and maintenance of blood cells that are formed from haematopoietic stem cells (HSCs) (18). Unlike HSCs, MSCs can be culture-expanded *ex vivo* in up to 40 or 50 cell doublings without differentiation (19). A dramatic decrease in MSC per nucleated marrow cell can be observed when the results are grouped by decade, thus showing a 100-fold decrease from birth to old age. Being pericytes present in all vascularized tissues, the local availability of MSC decreases substantially as the vascular density decreases by one or two orders of magnitude with age (20). In recent years, the discovery of MSCs with properties similar, but not identical, to BM-MSCs has been demonstrated in the stromal fraction of the connective tissue from several organs, including adipose tissue, trabecular bone, derma, liver and muscle (21-24). It is important to note that the origin of MSCs might determine their fate and functional characteristics (25). Studies of human bone marrow have revealed that about one-third of the MSC clones are able to acquire phenotypes of pre-adipocytes, osteocytes and chondrocytes (16). This is in concordance with data showing that 30% of the clones from bone marrow have been found to exhibit a trilineage differentiation potential, whereas the remainder display a bi-lineage (osteo-chondro) or uni-lineage (osteo) potential (26). Moreover, MSC populations derived from adipose tissue and derma present a heterogeneous differentiation potential; indeed, only 1.4% of single cells obtained from adipose-derived adult stem cell (ADAS) populations were tri-potent, the others being bi-potent or unipotent (27).

## 2. Effect of Mesenchymal Stem Cells on Immune cells

Mesenchymal Stem Cells have been shown to possess immunomodulatory characteristics, as described through the inhibition of T-cell proliferation *in vitro* (28-30). These observations have triggered a huge interest in the immunomodulatory effects of MSCs. The *in vitro* studies have been complemented *in vivo*, where both confirmed the immunosuppressive effect of MSC. MSC activating stimuli *in vitro*, appears to include the secretion of cytokines and the interaction with other immune cells in the presence of proinflammatory cytokines (Fig 1) (31). Primarily, the *in vivo* effect has been originally shown in a baboon model, in which infusion of *ex vivo*-expanded matched donor or third-party MSCs delayed the time to rejection of histo-incompatible skin grafts (29). The delay indicated a potential role for MSC in the prevention and treatment of graft-versus-host disease (GVHD) in ASCT, in organ transplantation to prevent rejection, and in autoimmune disorders. Recently, MSCs were used to successfully treat a 9-year-old boy with severe treatment-resistant acute GVHD, further confirming the potent immunosuppressive effect in humans (32). The immunosuppressive potential has no immunologic restriction, whether the MSCs are autologous with the stimulatory or the responder lymphocytes or the MSCs are derived from a third party. The degree of MSC suppression is dose dependent, where high doses of MSC are inhibitory, whereas low doses enhance lymphocyte proliferation in MLCs (33). Broadly, MSC modulate cytokine production by the dendritic and T cell subsets DC/Th1 and DC/Th2 (34), block the antigen presenting cell (APC) maturation and activation (35), and increase the proportion of CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells in a mixed lymphocyte reaction (36).



**Fig. 1. Potential mechanisms of the MSC interactions with immune cells.** Mesenchymal stem cells (MSCs) can inhibit both the proliferation and cytotoxicity of resting natural killer (NK) cells, as well as, their cytokine production by releasing prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO) and soluble HLA-G5 (sHLA-G5). Killing of MSCs by cytokine-activated NK cells involves the engagement of cell-surface receptors (Thick blue line) expressed by NK cells with its ligands expressed on MSCs. MSCs inhibit the differentiation of monocytes to immature myeloid dendritic cells (DCs), bias mature DCs to an immature DC state, inhibit tumour-necrosis factor (TNF) production by DCs and increase interleukin-10 (IL-10) production by plasmacytoid DCs (pDCs). MSC-derived PGE2 is involved in all of these effects. Immature DCs are susceptible to activated NK cell-mediated lysis. MSC Direct inhibition of CD4+ T-cell function depends on their release of several soluble molecules, including PGE2, IDO, transforming growth factor-β1 (TGFβ1), hepatocyte growth factor (HGF), inducible nitric-oxide synthase (iNOS) and haem-oxygenase-1 (HO1). MSC inhibition of CD8+ T-cell cytotoxicity and the differentiation of regulatory T cells mediated directly by MSCs are related to the release of sHLA-G5 by MSCs. In addition, the upregulation of IL-10 production by pDCs results in the increased generation of regulatory T cells through an indirect mechanism. MSC-driven inhibition of B-cell function seems to depend on soluble factors and cell-cell contact. Finally, MSCs dampen the respiratory burst and delay the spontaneous apoptosis of neutrophils by constitutively releasing IL-6.

### 3. Immunomodulatory effect of mesenchymal stem cells in innate immunity

Dendritic cells have the elementary role of antigen presentation to naïve T cells upon maturation, which in turn induce the proinflammatory cytokines. Immature DCs acquire the expression of co-stimulatory molecules and upregulate expression of MHC-I and II, as well as, other cell-surface markers (CD11c and CD83). Mesenchymal stem cells have profound effect on the development of DC, where in the presence of MSC, the percentage of DC with conventional phenotype is reduced, while that of plasmacytoid DC is increased, therefore biasing the immune system toward Th2 and away from Th1 responses in a PGE-2 dependent mechanism (37). Furthermore, MSCs inhibit the up-regulation of CD1a, CD40, CD80, CD86, and HLA-DR during DC differentiation and prevent an increase of CD40, CD86, and CD83 expression during DC maturation (38). When mature DCs are incubated with MSCs they have a decreased cell-surface expression of MHC class II molecules, CD11c, CD83 and co-stimulatory molecules, as well as, decreased interleukin-12 (IL-12) production, thereby impairing the antigen-presenting function of the DCs (Fig 1) (39, 40). MSCs can also decrease the pro-inflammatory potential of DCs by inhibiting their production of tumour-necrosis factor  $\alpha$  (TNF- $\alpha$ ) (40). Furthermore, plasmacytoid DCs (pDCs), which are specialized cells for the production of high levels of type-I IFN in response to microbial stimuli, upregulate production of the anti-inflammatory cytokine IL-10 after incubation with MSCs (34). These observations indicate a potent anti-inflammatory and immunoregulatory effect for MSC *in vitro* and potentially *in vivo*.

Natural killer (NK) cells are key effector cells of the innate immunity in anti-viral and anti-tumor immune responses through their Granzyme B mediated cytotoxicity and the production of pro-inflammatory cytokines (41). NK-mediated target cell lysis results from an antigen-ligand interaction realized by activating NK-cell receptors, and associated with reduced or absent MHC-I expression by the target cell (42). MSCs can inhibit the cytotoxic activity of resting NK cells by down-regulating expression of Nkp30 and natural-killer group 2, member D (NKG2D), which are activating receptors involved in NK-cell activation and target-cell killing (Fig 1) (43). Resting NK cells proliferate and acquire strong cytotoxic activity when cultured with IL-2 or IL-15, but when incubated with MSC in the presence of these cytokines, resting NK-cell, as well as, pre-activated NK cell proliferation and IFN- $\gamma$  production are almost completely abrogated (44, 45). It is worth noting that although the susceptibility of NK cells to MSC mediated inhibition is potent, the pre-activated NK cells showed more resistance to the immunosuppressive effect of MSC compared to resting NK cells (43). The susceptibility of human MSCs to NK-cell-mediated cytotoxicity depends on the low level of cell-surface expression of MHC class I molecules by MSCs and the expression of several ligands, that are recognized by activating NK-cell receptors. Autologous and allogeneic MSC were susceptible to lysis by NK cells (43), where NK cell-mediated lysis was inversely correlated with the expression of HLA class I on MSC (46). Incubation of MSCs with IFN- $\gamma$  partially protected them from NK-cell-mediated cytotoxicity, through the up-regulation of expression of MHC-I molecules on MSCs (43). Taken together, a possible dynamic interaction between NK cells and MSC *in vivo* exists, where the latter partially inhibit activated MSC, without compromising their ability to kill MSC, reflecting on an interaction tightly regulated by IFN- $\gamma$  concentration.

Neutrophils play a major role in innate immunity during the course of bacterial infections, where they are activated to kill foreign infectious agents and accordingly undergo a respiratory burst. MSCs have been shown to dampen the respiratory burst and to delay the

spontaneous apoptosis of resting and activated neutrophils through an IL-6-dependent mechanism (47). MSC had no effect on neutrophil phagocytosis, expression of adhesion molecules, and chemotaxis in response to IL-8, f-MLP, or C5a (47). Stimulation with bacterial endotoxin induces chemokine receptor expression and mobility of MSCs, which secrete large amounts of inflammatory cytokines and recruit neutrophils in an IL-8 and MIF-dependent manner. Recruited and activated neutrophils showed a prolonged lifespan, an increased expression of inflammatory chemokines, and an enhanced responsiveness toward subsequent challenge with LPS, which suggest a role for MSCs in the early phases of pathogen challenge, when classical immune cells have not been recruited yet (48). Furthermore, MSC have shown the capability to mediate the preservation of resting neutrophils, a phenomenon that might be important in those anatomical sites, where large numbers of mature and functional neutrophils are stored, such as the bone marrow and lungs (49).

#### 4. Immunomodulatory effect of mesenchymal stem cells in adaptive immunity

T-cells are major players of the adaptive immune response. After T-cell receptor (TCR) engagement, T cells proliferate and undergo numerous effector functions, including cytokine release and, in the case of CD8<sup>+</sup> T cells (CTL), cytotoxicity. Abundant reports have shown that T-cell proliferation stimulated with polyclonal mitogens, allogeneic cells or specific antigen is inhibited by MSCs (28, 29, 50-56). The observation that MSCs can reduce T cell proliferation *in vitro* is mirrored by the *in vivo* finding through infusions of *h*MSCs that control GVHD following bone marrow transplantation. Nevertheless, there is no demonstrable correlation between the measured effects of MSCs *in vitro* and their counter effect *in vivo* due to the lack of universality of methodology correlating the *in vitro* findings with the *in vivo* therapeutic potential.

MSC inhibition of T-cell proliferation is not MHC restricted, since it can be mediated by both autologous and allogeneic MSCs and depends on the arrest of T-cells in the G0/G1 phase of the cell cycle (55, 57). Thus, MSCs do not promote T-cell apoptosis, but instead maintain T cell survival upon subjection to overstimulation through the TCR and upon commitment to undergo CD95-CD95-ligand-dependent activation-induced cell death (57). MSC effects on T cell proliferation *in vitro* appear to have both contact-dependent and contact-independent components (58). Inhibition of T-cell proliferation by MSCs leads to decreased IFN- $\gamma$  secretion *in vitro* and *in vivo* associated with increased IL-4 production by T helper 2 (TH2) cells (34, 59). Taken together, there is an implication for a shift from a pro-inflammatory state characterized by IFN- $\gamma$  secretion to an anti-inflammatory state characterized by IL-4 secretion (Fig 1). An imperative role for effector T-cell is the MHC restricted killing of virally-infected or of allogeneic cells mediated via CD8<sup>+</sup> CTLs, and which is down-regulated by MSC (60).

Regulatory T cells (Tregs), a subpopulation of T cells, are vital to keep the immune system in check, help avoid immune-mediated pathology and contain unrestricted expansion of effector T-cell populations, which results in maintaining homeostasis and tolerance to self antigens. Tregs are currently identified by co-expression of CD4 and CD25, expression of the transcription factor FoxP3, production of regulatory cytokines IL-10 and TGF- $\beta$ , and ability to suppress proliferation of activated CD4<sup>+</sup>CD25<sup>+</sup> T cells in co-culture experiments. Differential expression of CD127 ( $\alpha$ -chain of the IL-7 receptor) enable flow cytometry-based separation of human Tregs from CD127<sup>+</sup> non-regulatory T-cells (61). MSCs have been

reported to induce the production of IL-10 by pDCs, which, in turn, trigger the generation of regulatory T cells (Fig 1) (34, 40). Furthermore, Tregs secrete TGF- $\beta$  and when used *in vitro*, TGF- $\beta$  in combination with IL-2 directs the differentiation of T-cells into Tregs, while Tregs suppress the proliferation of TCR-dependent proliferation of effector CD25<sup>null</sup> or CD25<sup>low</sup> T-cells in a non-autologous fashion. Also TGF- $\beta$  alters angiogenesis following injury in experiments using MSC (62). In addition, after co-culture with antigen-specific T-cells, MSCs can directly induce the proliferation of regulatory T-cells through release of the immunomodulatory HLA-G isoform HLA-G5 (Fig 1) (63). Taken together, MSCs can modulate the intensity of an immune response by inhibiting antigen-specific T-cell proliferation and cytotoxicity and promoting the generation of regulatory T-cells.

Antibody producing B-cells constitute the second main cell type involved in adaptive immunity. Interactions between MSCs and B-cells have produced controversial results attributable to the inconsistent experimental conditions used (31, 55, 64). Initial reports on mice suggested that MSC exercise a dampening effect on the proliferation of B-cells (64), which is in concordance with most published works to date (31, 55, 64). Furthermore, MSCs can also inhibit B-cell differentiation and constitutive expression of chemokine receptors via the release of soluble factors and cell-cell contact mediated possibly by the Programmed Cell Death 1 (PD-1) and its ligand (31, 64). The addition of MSCs, at the beginning of a mixed lymphocyte reaction (MLC), considerably inhibited immunoglobulin production in standard MLC, irrespective of the MSC dose employed, which suggests that third-party MSC are able to suppress allo-specific antibody production, consequently, overcoming a positive cross-match in sensitized transplant recipients (65). However, other *in vitro* studies have shown that MSCs could support the survival, proliferation and differentiation to antibody-secreting cells of B-cells from normal individuals and from pediatric patients with systemic lupus erythematosus (66, 67). A major mechanism of B-cell suppression was *h*MSC production of soluble factors, as indicated by transwell experiments, where *h*MSCs inhibited B-cell differentiation shown as significant impairment of IgM, IgG, and IgA production. CXCR4, CXCR5, and CCR7 B-cell expression, as well as chemotaxis to CXCL12, the CXCR4 ligand, and CXCL13, the CXCR5 ligand, were significantly down-regulated by *h*MSCs, suggesting that these cells affect chemotactic properties of B-cells (Fig 1). B-cell costimulatory molecule expression and cytokine production were unaffected by *h*MSCs (64). Regardless of the controversial *in vitro* effects, B-cell response is mainly a T-cell dependent mechanism, and thus its outcome is significantly influenced by the MSC-mediated inhibition of T-cell functions. More recently, Corcione et al have shown that systemic administration of MSCs to mice affected by experimental autoimmune encephalomyelitis (EAE), a prototypical disease mediated by self-reactive T cells, results in striking disease amelioration mediated by the induction of peripheral tolerance (52). In addition, it has been shown that tolerance induction by MSCs may occur also through the inhibition of dendritic-cell maturation and function (34, 35), thus suggesting that activated T cells are not the only targets of MSCs.

Low concentrations of IFN- $\gamma$  upregulate the expression of MHC-II molecules by MSCs, which indicates that they could act as antigen presenting cells (APCs) early in an immune response, when the level of IFN- $\gamma$  are low (68, 69). However, this process of MHC-II expression by MSCs, along with the potential APC characteristics, was reversed as IFN- $\gamma$  concentrations increased. These observations could suggest that MSCs function as conditional APC in the early phase of an immune response, while later switch into an immunosuppressive function (68). Since bone marrow might be a site for the induction of T-

cell responses to blood-borne antigens (70), and since MSC are derived from the stromal progenitor cells that reside in the bone marrow, therefore, MSC express a yet unidentified role in the control of the immune response physiology of the bone marrow. Dendritic cells are the main APC for T-cell responses, and MSC-mediated suppression of DC maturation would prohibit efficient antigen presentation and thus, the clonal expansion of T-cells. Direct interactions of MSCs with T-cells *in vivo* could lead to the arrest of T-cell proliferation, inhibition of CTL-mediated cytotoxicity and generation of CD4<sup>+</sup> regulatory T-cells. As a consequence, impaired CD4<sup>+</sup> T-cell activation would translate into defective T-cell help for B-cell proliferation and differentiation to antibody-secreting cells.

The *h*MSCs express few to none of the B7-1/B7-2 (CD80/CD86) costimulatory-type molecules; this appears to contribute, at least in part, to their immune privilege characteristic. Mechanisms that lead to immune tolerance rely on interrelated pathways that involve complex cross talk and cross regulation of T-cells and APCs by one another. Both soluble mediators and modulation exerted via complex networks of cytokines and costimulatory molecules appear to play a role in MSC regulation of T cells, and these mechanisms invoke both contact-dependent and -independent pathways.

Although many of the studies use MSC-conditioned medium, both contact-dependent and -independent mechanisms are probably invoked in the therapeutic use of MSCs (20, 71). In addition to cytokines, the network of costimulatory molecules is hypothesized to play a prominent role in modulating tolerance and inflammation. MSCs down-regulate the expression of costimulatory molecules (30, 72, 73). The discovery of new functions for B7 family members, together with the identification of additional B7 and CD28 family members, is revealing new ways in which the B7:CD28 family may regulate T-cell activation and tolerance. Not only do CD80/86:CD28 interactions promote initial T-cell activation, they also regulate self-tolerance by supporting CD4<sup>+</sup>CD25<sup>+</sup> Treg homeostasis (74-76). Cytotoxic T-lymphocyte antigen 4 (CTLA-4) can exert inhibitory effects in both B7-1/B7-2-dependent and -independent fashions. B7-1 and B7-2 can signal bi-directionally through engaging CD28 and CTLA-4 on T cells and by delivering signals into B7-expressing cells (77). The B7 family members—inducible co-stimulator (ICOS) ligand, PD-L1 (B7-H1), PD-L2 (B7-DC), B7-H3, and B7-H4 (B7x/B7-S1)—are expressed on professional APC cells, while B7-H4 and B7-H1 are expressed on *h*MSCs and on cells within non-lymphoid organs. These observations may provide a new means for regulating T-cell activation and tolerance in peripheral tissues (31, 71, 78). ICOS and PD-1 are expressed upon T-cell-induction, and they regulate previously activated T-cells (79). Both the ICOS:ICOSL pathway and the PD-1:PD-L1/PD-L2 pathway play a critical role in regulating T-cell activation and tolerance (79). There is consensus that both CTLA-4 and PD-1 inhibit T-cell and B-cell activation and may play a crucial role in peripheral tolerance (79, 80). Both CTLA-4 and PD-1 functions are associated with Rheumatoid Arthritis (RA) and other autoimmune diseases. PD-1 is over expressed on CD4<sup>+</sup> T cells in the synovial fluid of RA patients (81). Whether or not these costimulatory molecules are critical mediators of MSC-mediated immune suppression and/or tolerance *in vivo* is still under current investigation.

## 5. Mesenchymal Stem Cells escape the immune system *in vitro*

Studies have shown that MSCs escape the immune system, and this makes them a potential therapeutic tool for various transplantation procedures. MSCs express intermediate levels of HLA major histocompatibility complex (MHC) class I molecules

(16, 50, 82, 83), while they do not express HLA class II antigens of the cell surface. However, HLA class II is readily detectable by Western blot on whole-cell lysates of unstimulated adult MSCs, thus suggesting that MSCs contain intracellular deposits of HLA class II allo-antigens (83). Cell-surface expression can be induced by treatment of the cells with IFN- $\gamma$  for 1 or 2 days. Unlike adult MSCs, the fetal liver-derived cells have no intracellular nor cell surface HLA class II expression (84), but incubation with IFN- $\gamma$  initiated their intracellular expression followed by surface expression. Differentiation of MSCs into their mesodermal lineages of bone, cartilage, or adipose tissue, both in adult and fetal MSCs continued to express HLA-I, but not class II (84). Undifferentiated MSCs *in vitro* fail to elicit a proliferative response from allogeneic lymphocytes, thus suggesting that the cells are not inherently immunogenic (28, 30, 50). When pre-cultured with IFN- $\gamma$  for full HLA class II expression, MSCs still escape recognition by allo-reactive T-cells, (83, 84) as is the case with MSCs differentiated adipocytes, osteoblasts, and chondrocytes. Limited *in vivo* data demonstrate the persistence of allogeneic MSCs into immunocompetent hosts after transplantation. In one patient treated with MSCs, DNA of donor MSC could not be detected in any organ at autopsy few weeks after the infusion, while in another patient receiving MSCs from two donors, the donor DNA from both donors was detected in lymph node and colon, the target organs of GVHD, within weeks after infusion (85). Data from our lab indicated that MSC were undetectable after two weeks in an allogeneic system (86). Therefore, the question of whether MSCs are recognized by an intact allogeneic immune system *in vivo* remains open, although the *in vitro* data support the theory that MSCs escape the immune system. MSCs do not express FAS ligand or costimulatory molecules, such as B7-1, B7-2, CD40, or CD40L (50). When costimulation is inadequate, T-cell proliferation can be induced by the addition of exogenous costimulation. However, MSCs differ from other cell types, and no T-cell proliferation can be observed when they are cultured with HLA-mismatched lymphocytes in the presence of a CD28-stimulating antibody (50). However, in agreement with the *in vitro* data, infusion or implantation of allogeneic and MHC-mismatched MSCs into baboons has been well tolerated (87-89). Unique immunologic properties of MSCs were also suggested by the fact that engraftment of human MSCs occurred after intra-uterine transplantation into sheep, even when the transplantation was performed after the fetuses became immunocompetent (90). MSC mainly fail to activate T-cells and show to be targets for CD8<sup>+</sup> T cell-cytotoxicity, although controversial (60). Phyto-hemagglutinin (PHA) blasts, generated to react against a specific donor, will lyse chromium-labeled mononuclear cells from that individual but it will not lyse MSCs derived from the same donor. Furthermore, killer cell inhibitory receptor (KIR ligand)-mismatched natural killer cells do not lyse MSCs (60). Thus, MSCs, although incompatible at the MHC, tend to escape the immune system.

Although MSCs are transplantable across allogeneic barriers, a delayed type hypersensitivity reaction can lead to rejection in xenogenic models of human MSCs injected into immunocompetent rats (91). In this study, MSCs were identified in the heart muscle of severe compromised immune deficiency rats, in contrast to that of immunocompetent rats. In the latter group, peripheral blood lymphocytes proliferated after re-stimulation with human MSCs *in vitro*, thus suggesting cellular immunization. Such a proliferative response *in vitro* has not been detected in humans treated with intravenous (IV) infusion of allogeneic MSCs (Le Blanc and Ringdén, unpublished data, 2004).

## 6. Mechanisms of immunosuppression by Mesenchymal Stem Cells

Several studies have acknowledged the immunosuppressive activities of MSCs, but the underlying mechanisms are far from being fully characterized. The initial step in the interaction between MSCs and their target cells involves cell-cell contact mediated by adhesion molecules, in concordance with studies showing the dependence of T-cell proliferation on the engagement of PD-1 by its ligand (31). Several soluble immunosuppressive factors, either produced constitutively by MSCs or released following cross-talk with target cells have been reported, including nitric oxide and indoleamine 2,3-dioxygenase (IDO), which are only released by MSC after IFN- $\gamma$  stimulation with target cells (92, 93), and thus not in a constitutive manner. IDO induces the depletion of tryptophan from the local environment, which is an essential amino acid for lymphocyte proliferation. MSC-derived IDO was reported as a requirement to inhibit the proliferation of IFN- $\gamma$ -producing TH1 cells (92) and together with prostaglandin E2 (PGE-2) to block NK-cell activity (Fig 1) (44). In addition, IFN- $\gamma$ , alone or in combination with TNF- $\alpha$ , IL-1 $\alpha$  or IL-1 $\beta$ , stimulates the production of chemokines by mouse MSCs that attract T-cells and stimulate the production of inducible nitric-oxide synthase (iNOS), which in turn inhibits T-cell activation through the production of nitric oxide (56). It is worth noting that MSCs from IFN- $\gamma$  receptor (IFN- $\gamma$ -R1) deficient mice do not have immunosuppressive activity, which highlights the vital role of IFN- $\gamma$  in the immunosuppressive function of MSC (56).

Additional soluble factors, such as transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), hepatocyte growth factor (HGF), IL-10, PGE-2, haem-oxygenase-1 (HO1), IL-6 and soluble HLA-G5, are constitutively produced by MSCs (28, 34, 51, 63, 94) or secreted in response to cytokines released by target cells upon interacting with MSC. TNF- $\alpha$  and IFN- $\gamma$  have been shown to stimulate the production of PGE-2 by MSCs above constitutive level (34). Furthermore, IL-6 was shown to dampen the respiratory burst and to delay the apoptosis of human neutrophils by inducing phosphorylation of the transcription factor signal transducer and activator of transcription 3 (47), and to inhibit the differentiation of bone-marrow progenitor cells into DCs (95).

The failure to reverse suppression, when neutralizing antibodies against IL-10, TGF- $\beta$  and IGF were added to MLR reactions does point to the possibility that MSC may secrete as yet uncharacterized immunosuppressive factors (93). Galectin-1 and Galectin-3, newly characterized lectins, are constitutively expressed and secreted by human bone marrow MSC. Inhibition of galectin-1 and galectin-3 gene expression with small interfering RNAs abrogated the suppressive effect of MSC on allogeneic T-cells (Fig 1) (96). The restoration of T-cell proliferation in the presence of  $\beta$ -lactose indicates that the carbohydrate-recognition domain of galectins is responsible for the immunosuppression of T-cells and highlights an extracellular mechanism of action for the MSC-secreted galectins. In this respect, the inhibition of T-cell proliferation could result from either direct effects of galectin-1 and galectin-3 on T cells and/or through a direct or an indirect effect on dendritic cells (97).

HLA-G5 represents another important molecule involved in MSC mediated regulation of the immune response, where its production has been shown to suppress T-cell proliferation, as well as NK-cell and T-cell cytotoxicity, while promoting the generation of Tregs (63, 98). HLA-G protein expression is constitutive and the level is not modified upon stimulation by allogeneic lymphocytes in MSC/MLR. HLA-G5 is detected on MSCs by real-time reverse-phase polymerase chain reaction, immune-fluorescence, flow cytometry and enzyme-linked immunosorbent assay in the supernatant (99). Cell contact between MSCs and activated T-

cells induces IL-10 production, which, in turn, stimulates the release of soluble HLA-G5 by MSCs (63). It is worth noting that none of these molecules have an exclusive role and that MSC-mediated immune-regulation is a redundant system that is mediated by several molecules.

## 7. Mesenchymal Stem Cells in response to injury

One important characteristic of *h*MSCs is their ability to suppress inflammation resulting from injury, as well as, resulting from allogeneic solid organ transplants, and autoimmune disease. Consistent with *in vitro* studies, murine allogeneic MSCs are effective cellular therapy models in the treatment of murine models of human disease (52, 100-102). Several studies have documented the substantial clinical improvements observed in animal models, when MSC were systemically introduced as a therapy in mouse models of multiple sclerosis (102, 103), inflammatory bowel disease (104-106), infarct, stroke, and other neurologic diseases (107, 108), as well as diabetes (109). These findings strongly suggest that xenogeneic *h*MSCs are not immunologically recognized by various immunocompetent mouse models of disease and are able to home to sites of inflammation. However, the mechanisms behind the immunosuppressive actions at the site of inflammation and its association with the homing activity have not yet been completely elucidated.

Nitric Oxide (NO) mediate its effect partly through phosphorylation of Stat-5, which results in suppression of T- cell proliferation, partly through the inhibition of NO synthase or the inhibition of prostaglandin synthesis. This reveals the MSC-dependent effects on proliferation. Although indoleamine-2, 3-dioxygenase (IDO) has been hypothesized to be critical in mediating the effect of NO, neutralizing IDO by using a blocking antibody did not interfere with NO's suppressive effects (93, 110).

Within an *in vivo* setting, injury, inflammation, and/or foreign cells can lead to T-cell activation, which results in those T-cells producing proinflammatory cytokines including, but not limited to, TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\alpha$ , and IL-1 $\beta$ . Combinations of cytokines may also induce cell production of chemokines, some of which bind to CXCR3-R expressing cells (including T cells) that co-localize with MSCs. MSCs then produce NO, which inhibits Stat-5 phosphorylation, thereby leading to cell-cycle arrest (and thus halting T cell proliferation) (Fig 1) (110). In addition, iNOS appears to be important in mouse MSC *in vivo* effects. MSCs from mice that lack iNOS (or IFN- $\gamma$ R1) are unable to suppress GVHD. In contrast to mouse MSCs that use NO in mediating their immune-suppressive effect, *h*MSCs and MSCs from non-human primates appear to mediate their immune-suppressive effects via IDO (56). There is some controversy about whether the effect of IDO results from local depletion of tryptophan, or from the accumulation of tryptophan metabolites (which is suggested by data showing that use of a tryptophan antagonist, 1-methyl-L tryptophan, restored allo-reactivity that would otherwise have been suppressed by MSCs). In addition to its effect on the JAK-STAT pathway, NO may also influence mitogen activated protein kinase and nuclear factor  $\kappa$ B, which would cause a reduction in the gene expression of proinflammatory cytokines.

## 8. Mesenchymal Stem Cell clinical applications

The clinical experience with and the safety of MSCs is of utmost interest for their wide therapeutic applications. The pioneering *in vivo* studies with MSC focused on the

engraftment facilitation for the haematopoietic stem cells (111). Further work also focused on the regenerative functions of MSC in terms of functional repair of damaged tissues (112). Hypoimmunogenicity of MSC provided a critical advantage in their use for clinical and therapeutic purposes *in vitro* (50), followed by pre-clinical studies (29) and reaching the human clinical studies (32) with the use of allogeneic donors. Allogeneic MSC have proved to be an option with major advantages in clinical use, since the use of autologous MSC is hindered by the limited time frame for clonal expansion and the costly *in vitro* proliferation. However, some sub-acute conditions, such as autoimmune diseases, might allow the use of autologous MSCs and their culture *in vitro*. It is worth noting that some reports have recently challenged the belief that allogeneic MSCs are poorly immunogenic (113, 114), indicating that in some cases an autologous MSC source could be advantageous. Recent reports have shown that MSCs from patients with autoimmune disease have a normal capability to support hematopoiesis, (115) and to exercise immunomodulatory functions (116), and to show a normal phenotypic characteristics (117).

The perspective role of adult stem cells in degenerative disease conditions, where there is progressive tissue damage and an inability to repair, possibly due to the depletion of stem cell populations or functional alteration, has been considered. In cases of osteoarthritis, a disease of the joints where there is progressive and irreversible loss of cartilage characterized by changes in the underlying bone, Murphy et al showed that the proliferative capacity of the MSC was substantially reduced, and this was independent of the harvest site from patients with end-stage OA undergoing joint replacement surgery (118). In this study the marrow samples were harvested both from the site of surgery (either the hip or the knee) and also from the iliac crest. These effects were apparently disease-related, and not age-related. However, the data suggest that susceptibility to OA and perhaps other degenerative diseases may be due to the reduced mobilization or proliferation of stem cells. In addition, successfully recruited cells may have a limited capacity to differentiate, leading to defective tissue repair. Alternatively, the altered stem cell activity may be in response to the elevated levels of inflammatory cytokines seen in OA, which was confirmed by several other investigators (119, 120).

Similarly, the functional impairment of the anti-proliferative effect of MSCs derived from patients with aplastic anaemia (121) or multiple myeloma (122) might be resulting from an intrinsic abnormality in the microenvironment of the bone marrow, which is consistent with the possible use of autologous MSC for therapeutic purposes.

With the knowledge of the homing capacity of MSC and their capacity to engraft into the recipient's bone after systemic administration, MSCs have been utilized to treat children with severe osteogenesis imperfecta, leading to improved parameters of increased growth velocity and total body mineral content associated with fewer fractures (123). Systemic infusion of allogeneic MSCs also led to encouraging bone marrow recovery in patients with tumors following chemotherapy (123). The immunosuppressive effect of infused MSCs has been successfully shown in acute, severe graft-versus-host disease (GvHD) (32). The probable effect of MSC was the inhibition of donor T-cell reactivity to histocompatibility antigens of the recipient tissue. Currently, there is no successful therapy for steroid-refractory acute GVHD. The possible role of MSCs in this context is therefore of potential interest. Le Blanc et al reported a case of grade IV acute GVHD of the gut and liver in a patient who had undergone ASCT with cells from an unrelated female donor (32). The patient was unresponsive to all types of immunosuppression drugs. When the patient was infused with  $2 \times 10^6$  MSCs per kilogram from his HLA-haploidentical mother, his GVHD

responded with a decline in bilirubin and normalization of stools. After the MSC infusion, DNA analysis of his bone marrow showed the presence of minimal residual disease (124). When immunosuppression was discontinued, the patient again developed severe acute GVHD, with its associated symptoms within a few weeks.

Modulation of host allo-reactivity led to accelerated bone-marrow recovery in patients co-transplanted with MSCs and haplo-identical HSCs (125). Clinical trials are being conducted on the immunomodulatory potential of MSCs in the treatment of Crohn's disease, with the potential for those cells to contribute to the regeneration of gastrointestinal epithelial cells (126).

As described previously, MSCs are characterized by their hypoimmunogenicity. In 2000, data from several research groups demonstrated long-term allo-MSc engraftment in a variety of non-cardiac tissues in the absence of immunosuppression (88, 90). On the basis of these observations, investigators began to look into the possibility of allo-MSc engraftment into affected myocardium in rats, and later in swine, where allo-MSc were found to readily engraft in necrotic myocardium and favorably alter ventricular function (2). The allo-MSc engraftment occurred without evidence of immunologic rejection or lymphocytic infiltration in the absence of assisted immunosuppressive therapy emphasizing some of the apparent advantages of these cells over other cell populations for cellular cardiomyoplasty. The immunologically privileged status of MSCs was also observed in xenogeneic setting, where Saito et al injected MSC intravenously from C57BL/6 mice into immunocompetent adult Lewis rats (127). When these animals were later subjected to MIs, murine MSCs could be identified in the region of necrosis, and these cells expressed muscle specific proteins not present before coronary ligation.

## 9. Animal models

Consistent with results from *in vitro* studies, murine allogeneic MSCs are effective in the treatment of murine models of human disease (52, 103, 128). Several studies have reported clinical improvements in mouse models of multiple sclerosis and amyotrophic lateral sclerosis, inflammatory bowel disease, stroke, diabetes, infarct and GVHD using I.V. injected xenogeneic *h*MSCs rather than allogeneic MSCs (108, 109). A major advantage in using *h*MSCs in mouse models of human disease is that the possibility of gathering mechanistic data through measuring biomarkers from body fluids or using noninvasive imaging technology, which may prove to be an advantage in clinical studies when applied on humans.

In experiments designed to study the trafficking of *h*MSCs, investigators used mouse models of severe erosive polyarthritis characterized by an altered transgene allele that results in chronic over-expression of TNF- $\alpha$  and which resemble human RA patients (60, 72). The motive behind utilizing these mice models was to investigate similarities in MSC homing with mouse models of chronic asthma and acute lung injury. Injected *h*MSC revealed a reduction in ankle arthritis parameters associated with decrease appendage related erythema, possibly due to the MSC localization to ankle joints as revealed by bioluminescence (129). Similar observations for inducing tolerance were made using adipose-derived MSC, where Treg were induced in RA PBMC and in mouse models of arthritis (36, 130). Furthermore, studies of rheumatoid arthritis T-cells showed a down-regulation of effector response using adipose-derived MSCs (131). Variations in this potential described by the capability of MSCs to down-regulate collagen-induced arthritis,

and in the ability to induce Tregs, depend on the source of MSC (mouse vs. human) and its characteristics (primary isolate of MSC line), which reflect on difference in function compared to primary expanded MSC (132). Other studies reported that in the collagen-induced model of arthritis, mice infused with MSCs have increased numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells that express FoxP3 and thus reveal a Treg phenotype (20). Recent data on collagen-induced arthritis model, where murine MSCs did not reveal therapeutic benefits against arthritis *in vivo*, but did show anti-proliferative effect *in vitro* suggest that there is no appropriate *in vitro* measures that can be accurately extrapolated into a potential therapeutic utility of MSCs *in vivo*, and that mouse MSCs show difference in functional characteristics to *h*MSC in terms of immunoregulatory capacity (133).

MSC's immunological properties appeared to have potential therapeutic advantages in other forms of autoimmune diseases, especially in type 1 diabetes. In NOD mouse model, several physiological defects that aim to maintain peripheral and central tolerance contribute to the development of autoimmune diabetes. These defects are summed up as a combination of immune cell dysfunction (including T-cell, NK cells, B-cells, and dendritic cells), associated with the presence of inflammatory cytokine milieu (134). MSCs possess specific immunomodulatory properties capable of halting autoimmunity through immunomodulation processes described in this chapter. The processes might be through a direct effect via the presentation of differential levels of negative costimulatory molecules and the secretion of regulatory cytokines that affect regulatory T-cells/autoreactive T-cells. Furthermore, MSCs could modulate the immunological dysregulation observed in antibody producing B-cells and cytotoxic NK cells. Dendritic cells have been shown to be defective in NOD mice characterized by higher levels of costimulation with a potential capability to shift to a TH-1 type of immune response.

In an experimental mouse model of diabetes induced by streptozotocin, it was observed that MSCs promoted the endogenous repair of pancreatic islets and renal glomeruli (109). Similarly, co-infusion of MSCs and bone-marrow cells inhibited the proliferation of  $\beta$ -cell-specific T-cells isolated from the pancreas of diabetic mice and restored insulin and glucose levels through the induction of recipient-derived pancreatic  $\beta$ -cell regeneration in the absence of trans-differentiation of MSCs (135). These studies show that the *in vivo* administration of MSCs is clinically efficacious through the modulation of pathogenic  $\beta$ - and T-cell responses and through potent bystander effects on the target tissue.

The timing of MSC infusion seems to be a critical parameter in their therapeutic efficacy. In the EAE mouse model of multiple sclerosis, MSC systematically injected at disease onset ameliorated myelin oligodendrocyte glycoprotein (MOG)-induced EAE and further decreased the infiltration T-cells, B-cells and macrophages into the central nervous system (CNS). Furthermore, T cells isolated from the lymph nodes of MSC-treated mice did not proliferate after *in vitro* re-challenge with MOG peptide, which is an indication of the induction of T-cell anergy (52). Systematic injection of MSCs was found to inhibit the *in vivo* production of pathogenic plp-specific antibodies and to suppress the encephalitogenic potential of plp-specific T cells in passive-transfer experiments. In this model, the MSCs migrated to the lymphoid organs, as well as, to the inflamed CNS, where they exercised a protective effect on the neuronal axons *in situ* (135, 136). In these studies, the therapeutic effect of MSCs depended on the release of anti-apoptotic, anti-inflammatory and trophic molecules, as occurred in the case of stroke in rats (137), and, possibly, on the recruitment of local progenitors and their subsequent induction to differentiate into neural cells (138). As trophic effect, the MSCs appeared to favor oligo-dendrogenesis by neural precursor cells (139).

Several other studies have provided insights into the effects of MSCs mediated by cytokines. In a model of acute renal failure, the administration of MSCs increased the recovery of renal function through the inhibition of production of proinflammatory cytokines, such as IL-1 $\beta$ , TNF and IFN $\gamma$ , and through an anti-apoptotic effect on target cells (140). Along the same line, the anti-inflammatory activity of MSCs was revealed in a mouse model of lung fibrosis, where they inhibited the effects of IL-1 $\alpha$ -producing T cells and TNF-producing macrophages through the release of IL-1 receptor antagonist (IL-1RA) (141). The release of trophic factors such as the WNT-associated molecule secreted frizzled-related protein 2 (SFRp2), which leads to the rescue of ischemic cardiomyocytes and the restoration of ventricular functions represent another important function for MSC (142).

With all the promising therapeutic potential of MSC, there seems to be a growing concern about their association with tumors. The immunoregulatory and anti-proliferative effects of MSCs led to several studies investigating the inhibitory effect of MSCs on tumor growth. Inhibition or, more frequently, stimulation of tumor-cell proliferation *in vitro* and/or tumor growth *in vivo* by MSCs has been reported (143-145). The heterogeneous nature of the MSC populations and the different experimental tumor models used, contribute to the effect of tumors on MSC in which the microenvironment generated by tumors influence the behavior of MSCs (146). Two main mechanisms are probably involved in the enhancement of tumor growth by MSCs. First, the cell-to-cell cross-talk between MSCs and tumor cells contribute to tumor progression, thus integrating within the tumor stroma (147), and second, the suppressive effects of MSCs on the immune system of tumor-bearing hosts might facilitate tumorigenesis, as shown for the inhibition of melanoma rejection, possibly mediated by regulatory CD8<sup>+</sup> T cells (144). Irrespective of the possible interactions between cancer cells, immune cells and MSCs, the potential risk of stimulating the growth cancer by MSCs must be considered.

## 10. Conclusion

As a whole, the data accumulated from preclinical and clinical data indicate that bone marrow-derived MSCs have, in addition to their therapeutic purposes in regenerative medicine, effects that can result from other characteristics, such as their anti-proliferative and anti-inflammatory properties. The immuno suppressive activity of MSCs provides means for inducing peripheral tolerance following systemic injection mediated through the inhibition of cell division, thereby preventing their responsiveness to antigenic triggers while maintaining them in a quiescent state. In addition, the clinical efficacy of MSCs in different experimental model seems to occur almost only during the acute phase of disease associated with limited trans-differentiation, which indicates that the therapeutic effectiveness of MSCs relies heavily on their ability to modify microenvironments. These modifications occur through the release of anti-inflammatory cytokines, and anti-apoptotic and trophic molecules that promote the repair and protection of damaged tissues, as well as, maintain the integrity of the immune cells.

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