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REVIEW

Mechanisms of mesenchymal stromal cell immunomodulation

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Multipotent mesenchymal stromal cells (MSCs) have generated considerable interest in the fields of regenerative medicine, cell therapy and immune modulation. Over the past 5 years, the initial observations that MSCs could enhance regeneration and modulate immune responses have been significantly advanced and we now have a clearer picture of the effects that MSCs have on the immune system particularly in the context of inflammatory-mediated disorders. A number of mechanisms of action have been reported in MSC immunomodulation, which encompass the secretion of soluble factors, induction of anergy, apoptosis, regulatory T cells and tolerogenic dendritic cells. It is clear that MSCs modulate both innate and adaptive responses and evidence is now emerging that the local microenvironment is key in the activation or licensing of MSCs to become immunosuppressive. More recently, studies have suggested that MSCs have the capacity to sense their environment and have a role in pathogen clearance in conjunction with the resolution of insult or injury. This review focuses on the mechanisms of MSC immunomodulation discussing the multistep process of MSC localisation at sites of inflammation, the cross talk between MSCs and the local microenvironment as well as the subsequent mechanisms of action used to resolve inflammation.
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INTRODUCTION TO MULTIPOTENT MESENCHYMAL STROMAL CELLS (MSCs)

Although MSCs were first identified in the bone marrow over 40 years ago,[1–3] their intriguing capacity to modulate the immune response was only identified 30 years later with reports that MSCs could suppress T-cell proliferation *in vitro*[4–5] and prolong the survival of allogeneic skin grafts *in vivo*.[4] Since then our understanding of how MSCs mediate their immune-suppressive effects has significantly advanced. A large number of *in vitro* studies have provided insight into the effects of MSCs on both innate and adaptive immune responses. Specifically, MSCs have the capacity to interfere with many components of the innate immune system including complement, Toll-like receptor (TLR) signalling, macrophages, dendritic cells, neutrophils, mast cells and natural killer cells.[6–11] In regard to the adaptive immune response, MSCs can directly inhibit T-cell function, shift the T helper lymphocyte balance and induce functional regulatory T cells (Tregs).[12–14] Less is known about the effect of MSCs on B cells but some studies suggest that MSCs can also modulate B-cell proliferation and function.[15] Moreover, MSCs have been demonstrated to exert their anti-inflammatory effects in a number of *in vivo* models including graft versus host disease (GvHD), experimental autoimmune encephalomyelitis, inflammatory bowel disease and allergic airway disease.[10,12,16–18] Based on this wealth of data supporting an anti-inflammatory and pro-reparative role for MSCs, these cells have been used for the treatment of various inflammatory diseases in clinical trials (clinicaltrials.gov).

Although a small number of studies have identified the mechanisms involved in MSC protective effects, for the most part we do not fully understand how MSCs mediate their effect *in vivo*. Given that MSCs are already being utilised for the treatment of patients in clinical trials, it is imperative that the field gains a better understanding of exactly how MSCs mediate their effects in these different inflammatory disorders to ensure that MSC therapy can be utilised with optimal therapeutic efficacy and safety.

Considerable data support an anti-inflammatory effect of MSCs on immune cells, however, there are conflicting reports, which suggest that MSCs enhance immune cell survival and function. It is crucial that we delineate these disparate findings to ensure that MSC therapy does not exacerbate inflammatory disease. In the context of pathogenic insult or excessive sterile inflammation (inflammation in the absence of micro-organisms), it seems logical that MSCs would orchestrate the clearance of pathogens or necrotic cells associated with sterile inflammation through promotion of immune cell survival and function followed by resolution of inflammation through suppressive mechanisms. Combined with the idea that MSCs have the capacity to sense their environment, this suggests that MSCs are receptive to local biochemical signals and deploy a stepwise strategic approach to resolving inflammation and encouraging tissue repair.

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In this review, the major mechanisms of action mediated by MSCs in modulating inflammation and the capacity of MSCs to sense and react to their local microenvironment are discussed.

MSC MIGRATION TO SITES OF INFLAMMATION/INSULT/INJURY

The immune system is adept at recognising and responding to pathogenic insult[19] through specialised pathogen recognition receptors. These pathogen recognition receptors not only recognise pathogen-associated molecular patterns but also molecules associated with damaged cells and tissues (so called DAMPs) during sterile inflammation.[20] Following recognition, a signalling cascade triggers the release of complement components, acute phase proteins, pro-inflammatory cytokines and chemokines, which lead to the recruitment of innate immune cells such as neutrophils and macrophages.[21] It seems reasonable to assume that endogenous or adoptively transferred MSCs would also respond to these cues and migrate to the site of inflammation. However, some of the key limitations for MSC research have been the lack of specific markers and useful tracking studies to examine the migration and engraftment of MSCs *in vivo*. Currently, little is known about the capacity for MSCs to migrate to sites of tissue inflammation, and indeed in some cases MSCs have been shown to exert their immunosuppressive effect from a distance. For example, human bone marrow MSCs trapped in the lung microvasculature as microemboli secrete tumour necrosis factor (TNF)-α-stimulated gene/protein 6 (TSG-6), which suppresses the early immune response in murine models of myocardial infarction[22] and corneal injury.[23] MSCs have the capacity to migrate *in vitro* to a number of complement proteins, growth factors, cytokines and chemokines including complement component 1 subcomponent q (C1q),[24] C3a and C5a,[25] stromal cell derived factor-1 (SDF-1),[26,27] platelet derived growth factor AB, insulin like growth factor-1, epidermal growth factor and hepatocyte growth factor,[27] interleukin (IL)-1β,[28] TNF-α, CC chemokine ligand 5[29] and macrophage derived chemokine.[27] Moreover, short-term exposure to TLR-3 and 4 ligands significantly enhanced MSC migration *in vitro*,[29] and pre-stimulation of MSCs with TNF-α enhanced migration towards chemokines through upregulation of chemokine receptors.[27] *In vivo* studies have demonstrated that MSCs upregulated expression of CXCR4 and CCR7 and selectively migrated to ischaemic kidney in response to SDF-1α or to wounded skin in response to secondary lymphoid tissue chemokine *in vivo*.[26,30] Thus, it seems that MSCs have the capacity to migrate in response to signals produced by inflamed tissues and these signals may have a role in determining the function of MSCs, be that promotion of pathogen clearance or suppression of inflammation.

MSC LICENSING/ACTIVATION AT SITES OF INFLAMMATION

An emerging concept in the MSC field is that MSCs are not spontaneously immunosuppressive but require 'licensing' or activation to exert their immunosuppressive effects. In particular, interferon (IFN)-γ, TNF-α or IL-1β have been demonstrated to be required for the activation of MSCs to modulate immune responses.[18,31,32] Importantly, differential regulation of a number of MSC immunomodulatory molecules (including indoleamine 2,3-dioxygenase (IDO), prostaglandin E-2 (PGE-2), transforming growth factor (TGF)-β, TSG-6 and nitric oxide (NO)) by pro-inflammatory cytokines has been observed.[18,33] In addition to pro-inflammatory cytokines, TLR signalling has also been implicated in the licensing of MSCs. TLR3 and TLR4 activation of MSCs enhanced MSC immunosuppression *in vitro* through IDO induction via IFN-β and protein kinase R signalling.[34] In addition, TLR2 activation of human bone marrow MSCs resulted in the upregulation of the immune-suppressive protein galectin-3.[35] In contrast, Liotta *et al.*[36] observed the opposite effect, with TLR3 and TLR4 ligand binding leading to the downregulation of Jagged-1 and the failure of MSCs to modulate T-cell responses. The discrepancy in these studies may be resolved by the findings that TLR3 and TLR4 may differentially licence MSCs; with TLR4 priming inducing a pro-inflammatory phenotype and secretion of IL-6, IL-8 and TGF-β[29] a process reportedly enhanced by co-stimulation with IFN-γ.[37] In contrast, TLR3 priming induced anti-inflammatory MSCs (producing IDO, PGE-2, IL-4 and IL-1RA).[29] Further research is required on the effects of TLR activation on MSCs, however, these finding indicate that MSCs are receptive to environmental cues and may have the capacity to promote pathogen clearance or immune suppression. MSCs have the capacity to trigger complement activation, a process that would normally result in lysis of the complement activating cell. Surprisingly, high levels of C3 activation correlate with enhanced immuno-suppressive capacity of MSCs.[38] Importantly, MSCs express CD59, a complement regulatory protein, and also release complement factor H, which protects them from complement lysis.[38,39] Overall, it seems clear that the local microenvironment significantly influences MSC activation and immunoregulatory function (Figure 1).

MECHANISMS OF MSC IMMUNOMODULATION

MSCs possess an arsenal of immunosuppressive mechanisms, which can be deployed in the modulation of inflammation. Two very interesting paradigms have recently been proposed which postulate that (1) MSCs have sentinel functions that allow them to sense their microenvironment and act accordingly[40] and (2) MSCs become polarised towards either a pro-inflammatory phenotype or an immunosuppressive phenotype depending on the TLR signals received.[29] Together, these concepts help to resolve some of the conflicting data showing that in some cases MSCs enhance immune cell survival and function and in others they inhibit inflammation and encourage repair.

MSC immunomodulation takes place over a multistage process involving (1) MSC responsiveness to inflammation and possible migration to the site of tissue injury, (2) licensing or activation of MSCs, (3) promotion of pathogen clearance if required and (4) modulation of inflammation (Figure 1). As discussed earlier, MSCs may exert their immunosuppressive effects at a distance,[23,41] but many studies demonstrate that MSCs require contact with immune cells to exert their effects. Two very elegant studies have shown that mouse MSCs can also chemoattract T lymphocytes through the secretion of CXCL9, CXCL10,[18] and CCL2 (monocyte chemotactic protein-1).[12] The production of CXCL9 and CXCL10 by MSCs was induced by IFN-γ, TNF-α, IL-1α or IL-1β,[18] whereas CCL2 secretion by MSCs was regulated by the TNF receptor superfamily, member 6 (FAS).[12] Once MSCs have attracted effector T cells, this provides a platform for MSC contact with effector T cells and facilitates the direct immunomodulation of the T cells via production of NO by MSCs[18] or FAS/FASL (Fas ligand)-induced apoptosis.[12] Although differences have been reported in the mechanism of action by mouse and human MSCs,[32] the study by Akiyama *et al.*[12] definitively shows that the data obtained in the mouse model (demonstrating higher levels of apoptotic effector T cells and increased numbers of Treg as well as elevated serum levels of TGF-β) could also be observed in systemic sclerosis patients treated with MSCs, indicating that basic research findings on the mechanisms of action of MSCs can be extrapolated to the clinic.

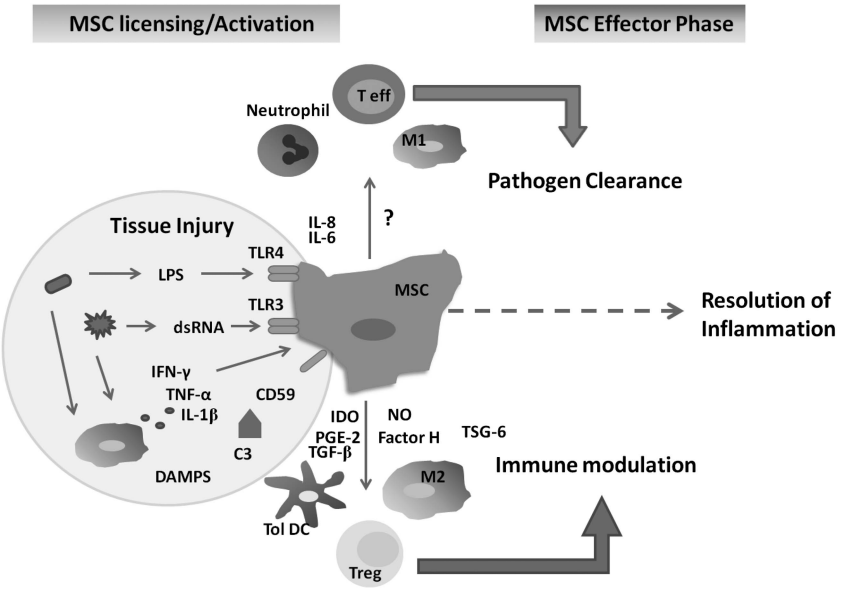

Figure 1 Activation by inflammatory mediators determines the effector mechanisms utilised by MSCs. MSCs are licensed/activated directly through TLR stimulation (pathogen-associated molecular patterns; (lipopolysaccharide (LPS), double-stranded RNA (dsRNA) and DAMPS) or indirectly by activated macrophages producing pro-inflammatory cytokines (IFN-γ, TNF-α and IL-1β) during tissue injury. MSCs have the capacity to trigger complement activation (C3), which in normal circumstances would target MSCs for complement lysis. However, complement activation of MSCs has been correlated with increased immunosuppressive activity. Importantly, MSCs express CD59 (a complement regulatory protein), and release complement factor H, which protects MSCs from complement lysis. Depending on the stimulus received, MSCs are thought to have the capacity to promote pathogen clearance or immune modulation (during the effector phase). MSCs may promote pathogen clearance through secretion of pro-inflammatory cytokines (IL-6 and IL-8), polarisation of pro-inflammatory M1 macrophages, anti-microbial activity,[100] through the enhanced survival and function of neutrophils or through other as of yet unidentified mechanisms. MSCs promote immune modulation through the secretion of immunosuppressive soluble factors (IDO, PGE-2, TGF-β, NO, TSG-6 and factor H among others), promotion of alternatively activated anti-inflammatory M2 macrophages, tolerogenic DC (Tol DC) or Treg. In response to pathogenic stimuli, MSCs may initially promote the clearance of pathogens followed by suppression of the immune response in the resolution of inflammation. A full colour version of this figure is available at the *Immunology and Cell Biology* journal online.

MSCs RE-EDUCATE MONOCYTES/MACROPHAGES IN THE CONTEXT OF TISSUE REPAIR

In response to activating signals, macrophages become polarised into either a classical M1 phenotype (pro-inflammatory, stimulated by TLR engagement or IFN-γ) or an alternative M2 phenotype (anti-inflammatory, stimulated by IL-4/IL-13). M1 macrophages are characterised by their high level production of pro-inflammatory cytokines including TNF-α and IL-1β whereas M2 are commonly associated with secretion of IL-10.[42] Given that macrophages have key roles at sites of inflammation,[21] it seems plausible that MSCs could interact with these cells and may even influence their polarisation. Indeed human MSCs derived from bone marrow, umbilical cord and cord blood have the capacity to modulate monocyte function *in vitro*.[7] Moreover, an array of studies demonstrate that MSCs alternatively activate macrophages, downregulating the production of TNF-α, IL-1α, IL-6 and IL-12p70 and increasing the production of IL-10 and enhancing the phagocytic activity.[6,9,10,43–45] In addition, two separate studies have shown that MSCs impair microglial activation and alternatively activate microglia to produce IGF-1, galectin-3 and to express factors associated with a neuroprotective phenotype.[46,47] Production of IDO and PGE-2 have been implicated in MSC modulation of macrophages,[43,44] and MSCs cultured in three-dimensional spheroids also have the capacity to reprogram macrophages through the production of PGE-2.[48] Perhaps the most notable studies are those that build a stepwise picture of how MSCs orchestrate macrophage polarisation and the influence the local microenvironment has on that process. First of these is the study by Nemeth *et al.*[10], in which MSCs ameliorate sepsis through alternative activation of macrophages. The authors elegantly elucidate the mechanisms of action, showing that LPS and TNF-α activate TLR4 and TNFR1 on MSCs to activate nuclear factor-κB signalling. This in turn leads to the expression of cycloxygenase (COX)-2 and synthesis of PGE-2 by MSCs, which bind EP2 and EP4 receptors on macrophages resulting in increased production of IL-10 and facilitating the resolution of inflammation. The second study utilised a zymosan-induced peritonitis model in which MSCs exerted anti-inflammatory effects through the production of TSG-6. In this case, zymosan, a TLR2 agonist, stimulated the activation of TLR2 and nuclear factor-κB signalling in macrophages. TNF-α secreted by the activated macrophages leads to the production of TSG-6 by MSCs. TSG-6 is then thought to limit TLR2/nuclear factor-κB signalling through direct interaction with CD44 expressed on the macrophage to initiate a negative feedback loop inhibiting the inflammatory response.[6] On the whole, micro-environmental cues present at the site of MSC activation seem to determine the particular mechanism of actions deployed by MSCs in modulating the immune response and resolving inflammation.

MSCs PRODUCE IMMUNOSUPPRESSIVE SOLUBLE FACTORS

MSC modulation of immune responses is mediated through an array of mechanisms, however, most of these mechanisms involve the production of immunosuppressive factors. The majority of these soluble factors are not constitutively produced by MSCs but in fact are induced through the licensing or activation of MSCs as described earlier. Herein, the key MSC-derived soluble factors and their modes of action are discussed.

INDOLEAMINE 2,3-DIOXYGENASE

IDO is an enzyme that catabolises tryptophan (an essential amino acid required for T-cell proliferation) into kynurenine metabolites that regulate T-cell proliferation.[49] MSC expression of IDO is induced by stimulation with IFN-γ[31,33,50] or through stimulation with TLR3 and TLR4 ligands, which involve the activation of protein kinase R, autocrine IFN-β signalling and activation of signal transducer and activator of transcription-1/interferon regulatory factor (STAT-1/IRF).[34] In contrast, Waterman *et al.*[29] showed that TLR3 but not TLR4 priming induced IDO production, while another study reported that TLR3 and TLR4 activation of MSCs abrogated the immunosuppressive effect of MSCs.[36] Differences in experimental set-up, timing, cell types and concentration of ligands may explain the differences observed, however, a clear definition of the precise parameters is still awaited. MSC-derived IDO has been associated with the re-education of immune cells including macrophage polarisation to the anti-inflammatory M2 phenotype,[43] induction of tolerogenic dendritic cells (DCs) and Treg *in vivo*[13] as well as promoting a Th1–Th2 switch.[13] In addition, MSC production of IDO directly impacts T-cell differentiation[51] and T and natural killer cell proliferation.[11,31,33,50] Blocking studies using the inhibitor 1-methyl-L-tryptophan or IDO knockout MSCs have demonstrated the important role for IDO in MSC suppression of these immune responses.[13,31,33,43,50,52] IDO is also known to have a role in microbial defence and it may be plausible that MSC-derived IDO could enhance microbial clearance as well as inhibition of immune responses.

PROSTAGLANDIN E-2

PGE-2 is a rapidly released and short acting small lipid mediator known to have a role in immune modulation. The pathway for prostaglandin synthesis involves the COX enzyme (COX-1 and COX-2) production of prostaglandin H2 from arachidonic acid, followed by conversion of prostaglandin H2 into prostaglandins via prostaglandin synthases.[53] COX-2 is constitutively expressed by MSCs and COX-2 inhibitor studies have shown that COX-2 is required for the production of PGE-2 by MSCs.[33,50,54] MSCs constitutively produce PGE-2 that is enhanced by stimulation with IFN-γ and TNF-α[33] as well as by TLR3 but not TRL4 ligands.[29] Furthermore, at least one study has identified that IL-6 is required for the production of PGE-2 and subsequent inhibition of inflammation in an experimental arthritis model,[55] however, this may be specific to an arthritic environment. A large body of data support the role of MSC-derived PGE-2 in the suppression of T-cell activation and proliferation both *in vitro* and *in vivo*.[33,50,56,57] In addition to T lymphocyte-specific effects, PGE-2 produced by MSCs has an important role in MSC reprogramming of macrophages[10,44,48] and DCs.[58] More recently, MSCs have been shown to inhibit mast cell function through a COX-2-dependent mechanism.[59] Importantly, the biochemical events involved in the mechanism of action of MSC-derived PGE-2 have been somewhat clarified by separate studies, which revealed that PGE-2 binds to either EP2 or EP4 on macrophages altering their phenotype[10] or to EP4 on $CD4^+$ T cells inhibiting Th17 differentiation.[54] Further elucidation of the interactions and signalling pathways involved will provide a better insight into how best to utilise this particular constitutively active property of MSCs in clinical application.

TNF-α STIMULATED GENE/PROTEIN 6

TSG-6 is an IL-1/TNF-inducible protein with anti-inflammatory properties. TNF-α stimulation of MSCs leads to the production of significant amounts of TSG-6. The observation that the majority of intravenously injected MSCs were found localised in the lung and not at the site of inflammation in a mouse model of myocardial infarction promoted the hypothesis that MSCs produced a potent anti-inflammatory agent that could act systemically. The molecule involved was identified as TSG-6 through microarray analysis of mouse lungs after infusion of human MSCs.[41] MSC-derived TSG-6 was shown to mediate protective effects in murine models of myocardial infarction,[41] corneal injury,[23] allogeneic corneal transplant[60] as well as zymosan-induced peritonitis.[6] In all models, TSG-6 inhibited the early inflammatory response, and specifically neutrophil infiltration and pro-inflammatory cytokines. Enhanced allogeneic corneal graft survival was associated with decreased activation of antigen-presenting cells in the graft and also in the draining lymph node.[60] The anti-inflammatory effect of TSG-6 in the corneal injury model was dose dependent and the inhibition of the early immune response significantly reduced the neovascularisation and subsequent development of opacity.[23] MSC-derived TSG-6 in the myocardial infarct model reduced infarct size and improved left ventricular function.[41] Clear evidence for the importance of MSC-derived TSG-6 was provided in systems using small interfering RNA knockdown or infusion of recombinant TSG-6 in place of MSCs. Of these studies, perhaps the zymosan-induced peritonitis model provides most insight into the cascade of events involved in MSC production of TSG-6 and subsequent suppression of macrophages as discussed above. Similar to the formation of microemboli in the lung,[6] MSCs can be cultured in spheroids *in vitro* and have been demonstrated to reprogram macrophages through production of PGE-2,[48] it may be interesting to examine a collaborative role for PGE-2 and TSG-6 *in vivo*.

NITRIC OXIDE

NO is produced as a result of the enzymatic reaction of inducible NO synthase and has the capacity to inhibit T-cell proliferation and induce T-cell apoptosis.[18] Stimulation of MSCs with IFN-γ and TNF-α- or IL-1-induced expression of inducible NO synthase in mouse MSCs.[18] NO is a potent molecule that mediates its effects at close proximity and two studies have elegantly shown that mouse MSCs lure T cells through the production of chemokines for subsequent suppression by local production of NO.[18,61] MSC-derived NO also induced apoptosis of alloreactive T cells through suppression of STAT-5 phosphorylation.[62] MSC production of NO enhanced cardiac allograft survival,[63] attenuated delayed-type hypersensitivity responses through induction of T-cell apoptosis[18,61] and prevented GvHD.[18] However, important species differences exist with regard to the role of NO in immune modulation. It may be that the importance of NO is more obvious in mouse MSCs compared with human.[32] Although NO may not be useful in MSC therapy in the clinic—the studies examining the role of NO have identified the very intriguing capacity of MSCs to chemoattract T cells to facilitate close contact allowing the provision of short acting molecules like NO.[18]

MSCs ALTER THE HELPER T-CELL BALANCE (Th1/Th2/Th17)

$CD4^+$ T helper cells become activated in response to pathogen- or danger-associated signals. Depending on the threat encountered $CD4^+$ T cells (Th0) differentiate into various T-cell subsets with distinct cytokine and gene expression profiles. The most common of these are the Th1, Th2 and Th17 subsets.[64,65] $CD4^+$ helper T cells have an essential role in host defence against pathogens, and for the most part, this component of the immune system is well regulated. However, in some circumstances, excessive immune responses can lead to significant tissue damage perhaps culminating in allergic or autoimmune diseases such as asthma, type-1 diabetes or multiple sclerosis.[16,66,67] MSCs have the capacity to modulate T-cell

proliferation and function[68–70] and in some cases MSCs mediate their protective effect through shifting the balance from Th1-driven responses to a more anti-inflammatory Th2 profile and vice versa for Th2-driven pathologies. Evidence from *in vitro* studies as well as *in vivo* models clearly demonstrate the ability for MSCs to shift the balance from a pro-inflammatory Th1 phenotype secreting IFN-γ and TNF-α to a more anti-inflammatory Th2 profile secreting increased levels of IL-4, IL-5, IL-10, IL-13 and the Th2 chemokine I-309.[66,67,71] The promotion of a switch towards a Th2 phenotype by MSCs was associated with delayed onset of type-1 diabetes in NOD mice[67] and amelioration of experimental autoimmune encephalomyelitis.[66] Contrastingly, in the context of allergic disease where Th2 cells drive allergic pathology, MSCs decrease the production of Th2-associated cytokines and enhance Th1 cytokine secretion[72,73] to create a greater balance and provide protection in allergic airway disease[72] and improve the symptoms of patients with sclerodermatous chronic GvHD.[73] MSCs also modulate Th17 differentiation[51,54] in favour of IL-4-producing Th2 cells or the generation of Treg.[66,74] Both IDO and PGE-2 have been implicated in MSC inhibition of Th17 differentiation,[51,68] and Duffy *et al.*[54] go a step further to elucidate the steps involved in this mechanism in their system. Specifically, contact-dependent COX-2 induction in MSCs leads to the production of PGE-2 and direct inhibition through EP4.[54] Importantly, MSCs can also mediate this effect through manipulation of the relative plasticity of T cells in suppressing the Th17 transcription factor retinoic-acid-receptor-related orphan receptor-γ t (RORγt) and upregulating Foxp3 to induce a Treg phenotype producing IL-10.[74] MSC production of the anti-inflammatory cytokine, TGF-β, has been shown to have a partial role in shifting the balance of Th1/Th2/Th17 and Treg in a rat model of experimental autoimmune myasthenia gravis.[14] Furthermore, MSCs re-educated Th1 cells acquired the capacity to inhibit T-cell proliferation *in vitro*.[74] Thus, there is now a considerable body of evidence to suggest that MSCs may provide protection in autoimmune and allergic diseases through shifting the balance of Th1/Th2 and Th17/Treg phenotypes.

MSCs INDUCE TOLEROGENIC DCs

The main function of dendritic cells is to act as sentinel cells and as such to present antigens activating antigen-specific helper T cells. These cells have a critical role in host defence and therefore in the generation of immune responses. MSCs can interfere with the development of both conventional and plasmacytoid DCs[75–77] but also with the key features of DC function; migration, maturation and antigen presentation[8] and an array of mechanisms have been implicated in these effects. Most notably, MSCs downregulate the expression of DC maturation markers including major histocompatibility complex (MHC) class II, CD40, CD80 and CD86[8,76–79] and modulate expression of the lymph node homing chemokine receptor CCR7 *in vitro*[8] and *in vivo*.[80] Moreover, MSC-mediated preservation of E-cadherin[8] expression by DCs fits with the concept that MSCs may prevent DC homing to the local lymph node. Interestingly, both soluble factors and contact-dependent signals have been identified in MSC modulation of DC maturation markers. MSC production of IL-6 has been shown to be involved in MSC downregulation of maturation markers.[8,76,77] Conversely, Li *et al.*[78] reported that IL-6 was not required and showed that contact-dependent Notch signalling was necessary for DC modulation. In support of a role for Notch signalling in this scenario, Zhang *et al.* found a partial role for contact-dependent Jagged-2 (a ligand of the Notch signalling pathway) signalling in the generation of regulatory DCs.[79] The importance of the original *in vitro* data on MSC modulation of CCR7 (English *et al.*[8]) and the subsequent hypothesis that MSCs inhibit DC migration to the lymph node *in vivo*,[69,70] has recently been supported.[80] Nevertheless, the exact mechanism utilised by MSCs to achieve this effect remains to be elucidated.

Akin to the effects of MSCs on macrophage polarisation discussed earlier, MSCs can also re-programme conventional lymphocyte stimulatory DCs into anti-inflammatory DCs with a tolerogenic phenotype.[13,58,78,79,81,82] DCs generated in the presence of MSCs produce higher levels of anti-inflammatory cytokines including IL-10 and lower levels of the pro-inflammatory cytokines IL-12 and TNF-α. The encounter with MSCs also results in enhanced phagocytic activity[79,82] typical of tolerogenic DCs. On a functional level, tolerogenic DCs generated by MSCs inhibited delayed-type hypersensitivity responses *in vivo*[79,82] and failed to induce activation of $CD4^+$ T cells (*in vitro* and *in vivo*).[58,80] Instead DCs that encountered MSCs promoted the generation of antigen-specific Treg *in vitro*.[78] The capacity of MSC educated DCs to induce a state of tolerance in the context of solid organ transplantation is a very exciting prospect and at least one *in vivo* study implicates MSC-induced tolerogenic DCs in kidney allograft survival in the presence of low-dose immunosuppression.[81] Although there are several observations that MSCs induce tolerogenic DCs as well as clear supporting evidence of the immunosuppressive or regulatory role played by these DCs, the intricate details of how MSCs induce these tolerogenic DCs is somewhat ambiguous. Given that IL-6 secreted by MSCs has been shown to be partially involved in the downregulation of DC maturation markers,[8,76,77] it initially seemed plausible that IL-6 might also be implicated in the generation of tolerogenic DCs. However, two independent studies did not find a role for IL-6 but instead demonstrated that PGE-2 and/or cell contact-dependent activation of the Notch signalling pathway were required for MSC induction of tolerogenic DCs.[58,78] A partial role for contact-dependent activation of Jagged-2 was also suggested by Zhang *et al.*[79] In further support of a contact-dependent mechanism, Chiesa *et al.*[80] propose that MSCs induce tolerogenic DCs through activation of AKT, which impaired nuclear factor-κB signalling, but could not find a role for secreted IL-10. Finally, a recent publication has shown that mouse embryonic fibroblast-derived MSCs generate a novel population of IL-10-dependent tolerogenic DCs through an IL-10-activated suppressor of cytokine signalling-3 (SOCS-3)-dependent mechanism.[82] Overall, the mechanisms of action mediated by MSCs in the generation of tolerogenic DCs are extremely varied and complex and no doubt are influenced by the context in which MSCs see DCs or DC precursors. Indeed, some of these variations may be explained by differences between *in vitro* and *in vivo* environments. The capacity for MSCs to induce tolerogenic DCs is uncontested, however, we must endeavour to ask the right questions and to fastidiously investigate the mechanisms of action involved for a greater understanding of how best to utilise MSCs in the clinic.

MSC INDUCTION OF TREG AND IMMUNE TOLERANCE

Tregs have an important role in the regulation of immune responses and in the prevention of autoimmune disease. Currently, there is significant interest in utilising Treg as a prospective therapy, in the setting of autoimmunity[83] and particularly organ transplantation[84,85] as Treg not only control allo- and autoreactive T-cell responses but also induce and maintain tolerance to self and non-self antigens. As previously discussed, MSCs favour the generation of Treg and this corresponds with a decrease in Th1, Th2 and Th17 lymphocytes.[12,14,74,86] The promotion of Treg by human bone

marrow-derived MSCs *in vitro* required cell–cell contact as well as PGE-2 and TGF-β[87] and these purified Treg were shown to functionally suppress alloreactive T lymphocyte proliferation *in vitro*.[87] In agreement with a role for cell contact, Selmani *et al.*[88] demonstrated that cell contact-dependent production of human leukocyte antigen-G5 was required for the expansion of Treg. Importantly, MSCs can induce the generation of antigen-specific Treg and TGF-β was identified as the key mechanism involved,[89] moreover, this study showed that MSCs inhibit experimental autoimmune uveitis in part through the generation of Tregs. MSC expansion or induction of Treg has been associated with protection from a number of alloreactive, autoimmune and allergic diseases including organ transplantation,[13,90–92] allergic airway disease,[16,93] type-1 diabetes[94,95] and inflammatory bowel disease.[12,96] The protection afforded by MSCs in these models is mediated through multiple mechanisms, which in the end lead to the induction or expansion of functionally active Treg and the postulated generation of tolerance.

The activation of MSCs to produce soluble factors is a shared observation among all of these studies, however, the sequence of events that lead to the generation of Treg and subsequent induction of tolerance is quite different and likely to be influenced by the particular microenvironment (allergic/alloreactive/autoreactive). TGF-β is the major soluble factor involved in MSC promotion of Treg *in vivo*.[12,14,93,95] Nemeth *et al.*[93] have incisively elucidated the steps involved in MSC generation of Treg in a mouse model of ragweed-induced asthma. The authors first established that MSCs remain in the lungs of allergic mice for longer periods than in healthy mice. Exposure of MSCs to IL-4 and IL-13 (typically produced in this allergic environment) induced bone marrow MSCs to produce TGF-β. A requirement for IL-4R/STAT-6 signalling was also required for MSC TGF-β production, in addition to another as yet unidentified factor. Perhaps a clue to this mysterious factor is provided by an earlier study identifying the requirement of TGF-β, PGE-2 and cell–cell contact, all acting within a nonredundant capacity.[87] Although the number of MSC present in the lung decrease over time, the opposite is true for Tregs and it may be that the initial effect of suppressing the pathogenic Th2 phenotype is mediated by MSCs, but MSC-induced Treg are the key cells for sustained control of allergic disease long after MSCs have disappeared.[93] As mentioned, the path to activated MSC-induced TGF-β production and subsequent Treg expansion/induction is likely to be varied and dependent on the system in question. Indeed, Th2-driven disease environments may have very different effects on MSCs in comparison with more Th1-associated autoimmune milieu. MSCs may promote a Th2 phenotype with decreases in the pro-inflammatory cytokines IL-17 and IFN-γ, but increases in IL-4, IL-10 and TGF-β in some models of autoimmune disease.[14,95,96] Although the mechanisms involved in these models may vary, neutralising studies demonstrated a role for TGF-β[14,95] or IL-10.[96] Akiyama *et al.*[12] have also identified a role for TGF-β-induced Treg in MSC protection against dextran sodium sulphate-induced colitis and fibrillin mutated systemic sclerosis. This study unravels a complex course of events starting with MSC induction of apoptosis of T effector cells by FAS/FASL facilitated by MSC–MCP-1 chemoattraction of T cells, followed by macrophage phagocytosis of the apoptotic cell debris and subsequent production of TGF-β resulting in the expansion of Treg.[12]

In the setting of organ transplantation, MSCs enhance allograft survival and in some cases induce tolerance associated with MSC promotion of Treg.[81,90,92] Similar to other findings, MSCs inhibited T-cell proliferation and promoted a Th2-dominant response while inducing tolerogenic DCs with attenuated allo-stimulatory capacity and decreasing anti-donor antibodies. These anti-inflammatory effects and the induction of tolerance maintaining Treg were mediated by MSC-derived IDO, as evidenced by the inability of IDO knockout MSCs to support these outcomes.[81] Finally, two separate studies have independently shown that depletion of Treg abrogates the anti-inflammatory effect mediated by MSCs[16,81] indicating an essential non-redundant role for Treg in the therapeutic mechanism.

OTHER MECHANISMS OF MSC IMMUNE MODULATION

Apart from the mechanisms discussed above, MSCs also exert a number of contact-dependent effects. Expression of the adhesion molecules vascular cell adhesion molecule 1 and intracellular adhesion moelcule 1 by MSCs are required for the contact-dependent interaction with T cells,[97] which facilitate MSC suppression of T cells through production of NO or IDO. A number of contact-dependent mechanisms involving FAS/FASL,[12] programmed death-1/programmed death ligand-1,[98] galectins,[99] CD39-induced adenosine[3] and Notch signalling[78,79] have also been reported in MSC immune modulation and some of these have been discussed above. Production of the anti-inflammatory cytokines IL-10 and TGF-β have also been reported with IL-10-promoting tolerogenic monocytes and macrophages[10,56] and TGF-β exerting their effects mainly in the context of Treg induction. However, Treg induction is not the only mechanism of MSC action *in vivo*, and Treg-independent effects have been described but require identification.[16] Further elucidation of the cascade of events and specific mechanisms, in the context of the particular environment, which MSCs are exposed to, will surely enhance our knowledge in this area and particularly with regard for cell contact-dependent mechanisms.

CONCLUSION AND FUTURE PERSPECTIVE

Significant progress has been made in the past 5 years in unravelling the complex series of events involved in MSC modulation of immune responses and the subsequent protection afforded in a range of disease models. Much of this progress has been made in animal models subsequent to clinical application, as has been the case for GvHD. However, the challenge now is to use existing pre-clinical and clinical data to inform better clinical trial design. Importantly, the relatively new concept that MSCs possess sensory-like characteristics and are influenced by their local environment may resolve some of the conflicting evidence that MSCs promote, or inhibit immune responses. Further research to define the influence of ongoing pathogenic infections, secondary to GvHD or organ transplantation (for example) on MSC activation and function will be essential. Overall, it appears that long-term engraftment or even localisation of MSCs (in some cases) at the site of injury is not required for MSC modulation of immune responses and pro-reparative effects. Moreover, a paradigm is now evolving that supports the idea that MSCs are receptive to environmental cues and have the potential to orchestrate the reprogramming of immune cells to promote host defence and/or resolve inflammation. Continued investigation of not only the ultimate mechanisms of action but also of the sequence of events involved in MSC-mediated protective effects and utilisation of this knowledge will enhance MSC therapeutic efficacy and benefit patients.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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