HUMAN GENE THERAPY 21:1641–1655 (December 2010)
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DOI: 10.1089/hum.2010.156

Review

Immunological Aspects of Allogeneic Mesenchymal Stem Cell Therapies

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Abstract

Allogeneic mesenchymal stem or stromal cells (MSCs) are proposed as cell therapies for degenerative, inflammatory, and autoimmune diseases. The feasibility of allogeneic MSC therapies rests heavily on the concept that these cells avoid or actively suppress the immunological responses that cause rejection of most allogeneic cells and tissues. In this article the validity of the immune privileged status of allogeneic MSCs is explored in the context of recent literature. Current data that provide the mechanistic basis for immune modulation by MSCs are reviewed with particular attention to how MSCs modify the triggering and effector functions of innate and adaptive immunity. The ability of MSCs to induce regulatory dendritic and T-cell populations is discussed with regard to cell therapy for autoimmune disease. Finally, we examine the evidence for and against the immune privileged status of allogeneic MSCs *in vivo*. Allogeneic MSCs emerge as cells that are responsive to local signals and exert wide-ranging, predominantly suppressive, effects on innate and adaptive immunity. Nonetheless, these cells also retain a degree of immunogenicity in some circumstances that may limit MSC longevity and attenuate their beneficial effects. Ultimately successful allogeneic cell therapies will rely on an improved understanding of the parameters of MSC–immune system interactions *in vivo*.

Introduction

A paradigm shift has occurred in our concept of how cell therapies utilizing mesenchymal stem cells (MSCs) mediate their beneficial effects. It is now appreciated that, although MSCs can be described as having trilineage differentiation potential and express a particular collection of surface markers, their effector function is based less on *in situ* differentiation, trans-differentiation, or fusion and more on paracrine effects and cross-talk with other cells present within diseased tissues. This concept of a trophic ("nourishing") effect of MSCs can be traced back to work carried out by Caplan and co-workers (Haynesworth *et al.*, 1996; Caplan and Dennis, 2006) and is also linked to earlier literature demonstrating the ability of bone marrow stromal cells to support hematopoiesis (Friedenstein *et al.*, 1974). More recently, the identification of a perivascular origin for MSCs from multiple organs has further enhanced the view of MSCs as cells with supportive and trophic functions during perturbations of tissue integrity (Sacchetti *et al.*, 2007; Crisan *et al.*, 2008; Caplan, 2009).

These insights are of particular significance to the development of MSCs as modulators of localized tissue inflammation and as therapeutic agents for immune-mediated diseases. Beginning approximately 10 years ago with *in vitro* co-culture experiments and progressing, more recently, to sophisticated *in vivo* models of immune/inflammatory disease, a clear and compelling profile of MSCs has developed as potent modifiers of a wide range of targets within the innate and adaptive arms of the immune system (Barry *et al.*, 2005; Uccelli *et al.*, 2008). The recognized clinical potential of MSC immunomodulatory effects now encompasses acute myocardial ischemia, stroke, kidney injury, inflammatory bowel disease, graft-versus-host disease (GVHD), multiple sclerosis, diabetes mellitus, and organ transplantation (Uccelli *et al.*, 2008; Caplan, 2009). Initial clinical trials have been completed or are underway in several of these areas (Ankrum and Karp, 2010). One important concept that has carried through from basic and preclinical studies to human clinical trials is that of the immune privileged status of MSCs transferred into an allogeneic host (Heng *et al.*, 2009). Stated in its simplest form, this concept implies that allogeneic MSCs (allo-MSCs) fail to activate the innate or elicit the adaptive cellular (T-cell) or humoral (B-cell/antibody) immune responses that typically result in rapid rejection of

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allogeneic cells and organs transplanted into a host in the absence of additional immunosuppressive therapy (Kahan, 2003). This would imply that allo-MSCs, prepared and stored in advance for "off-the-shelf" therapy, are of equal efficacy to individually prepared autologous MSC (auto-MSC) cultures and can be repeatedly administered without losing potency because of immune sensitization. A further extrapolation of some of the current literature would also suggest that allo-MSCs promote active immunological tolerance to "donor" major histocompatiblity complex (MHC) and other alloantigens—a property that could specifically enhance their therapeutic value in organ and tissue transplantation.

Although early, uncontrolled clinical case series provided exciting evidence of therapeutic benefits, the resulting optimism for off-the-shelf allo-MSC therapy has become tempered by the outcomes of recent, larger clinical trials in which allo-MSC products proved disappointing in terms of efficacy despite achieving safety endpoints (Ankrum and Karp, 2010). Thus, the time is right to ponder the route ahead for MSC-based cell therapies—particularly those based upon the large-scale expansion of cells from allogeneic donors. Most immunologists treated early descriptions of non-rejection of allo-MSCs or xenogeneic MSCs with some suspicion, but it is now the case that many thousands of patients have safely received allo-MSC therapies, and a large body of preclinical data has accumulated in support of the capacity of MSCs to modulate diverse immune processes *in vivo* (Uccelli *et al.*, 2008). Despite this, the more recent clinical results indicate that a more critical analysis of the interaction between allogeneic cell therapies and the recipient immune system will be essential for the rational development of commercially attractive MSCs and MSC-like products in the future (Ankrum and Karp, 2010).

Several questions present themselves for which definitive answers remain elusive: (1) What is the hierarchy of the immune suppressive functions of MSCs? (2) How much redundancy exists among the many suppressive processes that have been identified to date? (3) What are the limits of MSC immune modulation? (4) Are cellular and humoral components of the allo-immune response equally suppressed by MSCs? (5) Can the longevity and efficacy of allo-MSC therapies be enhanced through further suppression of allo-immune responses? In this article we consider the major mechanisms that convey immune modulatory properties to MSCs and examine the evidence for and against the immune privileged status of allo-MSCs *in vivo*. Along the way we discuss disease targets for which MSCs may be most suitable and highlight the hurdles that remain to be overcome in translating the promising laboratory studies into mainstream clinical practice.

MSC Suppression of Innate Immunity

The first encounter for any cell therapy upon delivery is with the components of the innate immune system that provide an effective antimicrobial defense but also a barrier to allogeneic and xenogeneic transplantation. It is clear that allo-MSCs (and indeed some xenogeneic MSCs) avoid acute and hyperacute rejection mechanisms normally mediated through the complement system. This is achieved through secretion of Factor H (Tu *et al.*, 2010) and most likely supported by MSC expression of the complement control proteins CD55, CD46, and CD59 (Komoda *et al.*, 2010) (B.P.M., unpublished data). Thus MSCs are protected from frontline deletion mechanisms operating in other tissue and cell transplant scenarios. However, MSCs are not inert to innate immune signaling, and there is evidence that MSCs are recruited by the anaphylatoxins C3a and C5a (Schraufstatter *et al.*, 2009), suggesting that they are attracted to and activated at sites of tissue damage rather than deleted.

The interaction of MSCs with natural killer (NK) cells has received little attention to date, particularly in the *in vivo* setting. Initial *in vitro* observations suggested that human MSCs were not susceptible to lysis by freshly isolated allogeneic NK cells and that MSCs inhibited NK cell secretion of interferon (IFN)-γ (Rasmusson *et al.*, 2003; Aggarwal and Pittenger, 2005). Subsequently, it has been demonstrated that, although human MSCs suppress proliferation, surface receptor expression, and effector functions of NK cells via prostaglandin E_2 (PGE_2) and 2,3-indoleamine dioxygenase, they can be lysed by activated NK cells (Poggi *et al.*, 2005; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2006, 2008). In the case of MSC interactions with neutrophils, the experimental evidence is even more limited. Of interest, however, is that Raffaghello *et al.* (2008) recently reported that human bone marrow–derived MSCs inhibited both apoptosis and the oxidative burst of resting and activated neutrophils while preserving their phagocytic and chemotactic functions. Although more work needs to be done in this area, the results fit with a model whereby MSCs modify ("reprogram") the functional properties of innate immune mediators in a manner that can both protect the MSC from frontline deletional mechanisms and broadly suppress a range of potentially destructive inflammatory pathways.

Recent studies of the influence of ligands of Toll-like receptors (TLRs) have reinforced the concept of MSCs as cells responsive to and modulatory of innate immunity (Pevsner-Fischer *et al.*, 2007; Liotta *et al.*, 2008; Tomchuck *et al.*, 2008; Opitz *et al.*, 2009; Z.J. Wang *et al.*, 2009). It is now clear that MSCs express a range of TLRs and that signaling via these receptors influences migration, survival, differentiation, and immunosuppressive capacity. Some studies have observed that MSC immune modulation can be downregulated by TLR3 and TLR4 ligands (Liotta *et al.*, 2008; Romieu-Mourez *et al.*, 2009) but enhanced by IFN-γ (English *et al.*, 2007). This suggests that MSCs may be particularly effective in suppressing chronic inflammation seen in autoimmunity (not driven by pathogens) without impairing inflammatory responses essential to antimicrobial defense (where TLR ligands would be abundant). There is also evidence, however, that TLR ligation in MSCs results in altered patterns of induction of cytokines and other inflammatory mediators that may, under some conditions, further enhance MSC immune suppressive properties (Tomchuck *et al.*, 2008; Lombardo *et al.*, 2009). In the future, it will be interesting to determine whether this increased attraction of innate immune cells by TLR-activated MSCs represents a barrier to therapeutic immune modulation or, in fact, facilitates anti-inflammatory cell–cell interactions. Overall, the impact of TLR ligation on MSC functions (and immunogenicity) *in vivo* is incompletely understood at present and may prove to be a key modifiable factor for optimizing the clinical benefits of allo-MSCs.

Another important element of the influence of MSCs on innate immunity relates to the interaction with monocytes

and monocyte-derived inflammatory cells. Evidence is accumulating that monocytes and macrophages may be "programmed" by their surrounding microenvironment either to mediate potent, locally destructive and lytic effects (perhaps appropriate for immediate clearance of dead cells and prevention of infection at a site of injury) or to produce a range of anti-inflammatory, pro-regenerative factors (indicative of a central role in the resolution and repair phase of tissue injury) (Tesar, 2008; Stout *et al.*, 2009; Geissmann *et al.*, 2010). Several recent studies have provided direct evidence that MSCs are involved in this programming (Ohtaki *et al.*, 2008; Kim and Hematti, 2009; Nemeth *et al.*, 2009). Most notably, Nemeth *et al.* (2009) demonstrated convincingly that both auto- and allo-MSCs reduced mortality from sepsis in a mouse model through a direct interaction with macrophages in the lung that resulted in enhanced production of interleukin (IL)-10 and was mediated by a complex monocyte/MSC cross-talk involving TLRs, tumor necrosis factor (TNF), nitric oxide, and PGE_2. Taken together with the previously discussed literature on MSC interactions with the complement system, NK cells, neutrophils, and ligands for pattern recognition receptors or TLRs, these studies paint a striking picture of the complexity of MSC cross-talk with the innate immune system and of the rich potential for harnessing these effects for therapeutic benefits.

MSC Influence on Dendritic Cells

Dendritic cells (DCs) play a critical role in adaptive immunity acting as the primary antigen-presenting cell to initiate antigen-specific $CD4^+$ helper T cells. This function has been extensively reviewed elsewhere (Steinman and Banchereau, 2007), but a simplified summary is useful to convey the importance of the MSC–DC interaction. There are a variety of specialized DC subsets with considerable flexibility in development such that precursors with myeloid or lymphoid characteristics can be identified. Some lymphoid tissues generate conventional but non-migratory DC locally from precursors also found in bone marrow (Naik *et al.*, 2007), whereas conventional migratory DCs are generated in the bone marrow from hematopoietic pro-DC precursors often via a monocytic intermediate (Fig. 1). On differentiation, conventional bone marrow–derived DCs expressing the $\alpha_x\beta_2$ integrin (CD11c:CD18) and C-C chemokine receptor (CCR) 6 migrate to peripheral tissues (Fig. 1) (Cook *et al.*, 2000; Kucharzik *et al.*, 2002; Osterholzer *et al.*, 2005). Such immature DCs (iDCs) can be found within skin (Langerhans cells), interstitial and epithelial tissues where they express tissue-anchoring E-cadherin and perform sentinel functions. iDCs have the capacity to take up antigen through phagocytosis and macropinocytosis and to process antigen for loading onto MHC class II molecules; thus iDCs can be considered to perform antigen acquisition functions in the periphery (Steinman and Nussenzweig, 2002; Steinman and Banchereau, 2007). On antigen encounter, iDCs undergo a process termed maturation that sees a remarkable alteration in biological activity, to become mature DCs (mDCs). mDCs downregulate CCR6 and E-cadherin but express CCR7, resulting in chemotaxis to local secondary lymphoid tissues such as the lymph nodes to fulfill their role in antigen display to the adaptive immune system (Iwasaki and Kelsall, 2000). Maturation is accompanied by the expression of the naive

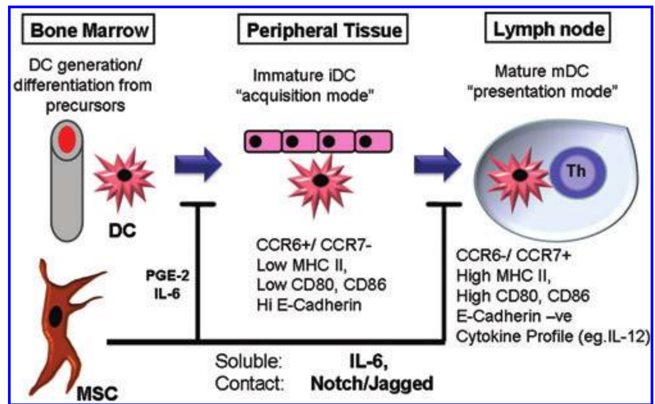

FIG. 1. MSCs modulate DC function. DCs are the primary initiators of adaptive T-cell immunity by acting as professional antigen-presenting cells; consequently they contribute to allogeneic cell rejection. *In vitro* studies suggest that allogeneic MSCs interfere with immature DC generation and maturation through contact-dependent and -independent processes resulting in a tolerogenic phenotype. Color images available online at www.liebertonline.com/hum.

T-cell chemoattractant C-C chemokine ligand (CCL) 18 and upregulation of MHC class II and the co-stimulatory molecules CD80 and CD86, among others (Masten *et al.*, 1997). Thus the mDC in the lymph node is in "antigen presentation mode" and ideally placed to initiate and expand antigen-specific $CD4^+$ helper T cells (Fig. 1). This process is essential to initiate adaptive immunity against foreign antigen, but of course if DCs derive from a non-MHC identical transplanted organ, graft, or transfusion, then conventional DCs will promote T-cell-mediated alloreactivity and consequent rejection (Morelli and Thomson, 2003). Conversely, antigen presentation in the absence of costimulation (CD80/CD86, etc.) can result in T-cell non-responsiveness or anergy, and DCs with immature or semi-mature phenotypes are now thought to play a role in peripheral tolerance induction (Turnquist and Thomson, 2008). These processes are thus essential to the initiation of alloreactivity, autoimmunity, and tolerance.

A distinct lineage of plasmacytoid DCs (pDCs) can also be derived from a common proDC precursor (Naik *et al.*, 2007; Shortman and Naik, 2007) and are identified by expression of CXCR3 and BDCA-2 (human) or Siglec-H (mouse). Although pDCs appear to be less effective in supporting the expansion of naive antigen-specific T cells, they play an important role in sustaining conventional DC production of IL-12 and in the detection and amplification of antiviral responses through TLR7 and TLR9, leading to type I IFN production and pro-inflammatory cytokine release (Liu, 2005; Zucchini *et al.*, 2008).

Given the central role of DCs in allogeneic rejection and the powerful allosuppressive influence of MSCs, it is not surprising that the interaction of MSCs with DCs has been the focus of much attention. Clearly MSCs modulate different aspects of DC function *in vitro* (Zhang *et al.*, 2004; Djouad *et al.*, 2007; English *et al.*, 2008; Zhang *et al.*, 2009), and this has a functional counterpart *in vivo* (H. Li *et al.*, 2008; Popp *et al.*, 2008; Rossignol *et al.*, 2009; Li *et al.*, 2010). It is worthwhile separating the functions related to DC generation and DC maturation as these events may occur in different

anatomical locations and concern distinct biological functions. First, it is clear that MSCs influence DC development. This is not surprising as both MSCs and many DC precursors are bone marrow residents and MSCs play a role in conditioning the niche for hematopoiesis (Dazzi *et al.*, 2006; Sacchetti *et al.*, 2007; Morikawa *et al.*, 2009). At the level of development, MSC co-culture strongly inhibits the initial differentiation of monocytes to iDCs *in vitro* (Beyth *et al.*, 2005; Nauta *et al.*, 2006a). This effect is reversible (Beyth *et al.*, 2005) and can be replicated by MSC-derived soluble factors, including PGE_2 and IL-6 (Djouad *et al.*, 2007). It is interesting that MSCs seem to have differential effects on the generation of conventional DCs (suppression) and pDCs (no suppression) (Chen *et al.*, 2007). The implications of these observations require careful interpretation, but it may indicate that MSCs have suppressive effects that can be bypassed when antiviral responses are required for protection—an interpretation supported by recent work (Karlsson *et al.*, 2008).

The major function of DCs in the epithelial tissues is to act as sentinels and, upon maturation, to initiate cell-mediated immunity. There are now consistent data from several sources showing that MSCs modulate or interfere with DC maturation in both the mouse and the human (Zhang *et al.*, 2004; Djouad *et al.*, 2007; Jung *et al.*, 2007; English *et al.*, 2008; Magatti *et al.*, 2009; van den Berk *et al.*, 2009; Zhang *et al.*, 2009). DCs exposed to maturation factors such as lipopolysaccharide or TNF-α co-cultured with MSCs failed to show regular upregulation of maturation markers such as MHC class II, CD40, or CD86 costimulatory molecules (Djouad *et al.*, 2007; English *et al.*, 2008). Similar effects have been seen with MSCs from amniotic, umbilical cord, or adipose sources (Wang *et al.*, 2008; Magatti *et al.*, 2009; van den Berk *et al.*, 2009; M. Wang *et al.*, 2009).

The encounter of DCs with MSCs abrogates the capacity of antigen-pulsed DCs to support cognate $CD4^+$ T-cell proliferation (English *et al.*, 2008). This extends to allo-recognition as well. Allo-MSCs suppress DCs from presenting (MHC-derived) allo-antigen, thus suppressing a major pathway of allo-recognition (English *et al.*, 2008). MSCs also prevent loss of iDC E-cadherin expression, prevent upregulation of CCR7, and inhibit chemotactic ability of DCs (English *et al.*, 2008). Thus MSCs suppress maturation marker expression, antigen presentation capability, and capacity to respond to lymph node–derived chemotactic signals—the three cardinal features of conventional DC maturation. Unlike suppression of T-cell proliferation in mixed lymphocyte reaction (MLR), both contact-dependent and soluble factors contribute to this immunomodulatory phenomenon. The contact-dependent signal appears to involve members of the Notch-Jagged signaling pathway (Y.P. Li *et al.*, 2008; Zhang *et al.*, 2009) (L. Tobin, personal communication), whereas MSC-derived IL-6 contributes to the soluble signal (Djouad *et al.*, 2007; English *et al.*, 2008) (Fig. 1).

The consequence of this immune modulation is that DCs may display an altered profile of cytokine expression (Aggarwal and Pittenger, 2005) with reduced IL-12 (Zhang *et al.*, 2004; Jiang *et al.*, 2005) or increased IL-10 (Aggarwal and Pittenger, 2005) production, adopt a tolerogenic capacity (H. Li *et al.*, 2008; Y.P. Li *et al.*, 2008; Popp *et al.*, 2008), and become capable of an indirect suppression through induction of regulatory T (Treg) cells (Beyth *et al.*, 2005). The functional significance of MSC modulation of DCs *in vivo* is difficult to assess, and there is an urgent need for more focus on this topic. However, MSCs can alter the migratory property of DCs to delay the development of murine lethal acute GVHD (H. Li *et al.*, 2008) and can suppress DC function during allogeneic islet transplant in a diabetic model (Kim *et al.*, 1997). These characterizations of MSC function have considerably extended our understanding of MSC-mediated immune suppression beyond the limited understanding achievable from MLR studies to suggest that DC modulation is a major pathway of MSC immune suppression.

MSC Induction of Treg Cells

There are numerous mechanisms by which the adaptive immune system achieves tolerance to self-antigen, and understanding these offers an opportunity to develop new interventions against autoimmunity and to prevent rejection of allografts. The central mechanism of tolerance is the deletion of self-reactive lymphocytes in the thymus (T cells) and the bone marrow (B cells) (Peterson *et al.*, 2008; Irla *et al.*, 2010). The mechanisms of peripheral tolerance support central tolerance, and among these are suppressor actions mediated by a group of cells loosely termed Treg cells. This term encompasses a variety of cells of different phenotypes and lineages, including Tr1 cells, T-helper (Th) 3 cells, $CD8^+$ suppressor cells, NK-like cells, and some $\gamma\delta$T cell populations (Tang and Bluestone, 2008). The two principal suppressor populations are considered to be $CD4^+$ $CD25^{high}$ $FOXP3^+$ T cells that develop in the thymus (sometimes called natural Treg) and T cells that can develop from naive T cells in the periphery (termed inducible or adaptive Treg). The latter may also express the FOXP3 transcription factor. A full discussion of the role of Treg cells is not possible in the current context, but there is abundant evidence that Treg cells play a central role in suppressing a range of autoimmune disease and that loss of functional FOXP3 results in fatal multiorgan autoimmunity (Brunkow *et al.*, 2001; Lin *et al.*, 2005; Lahl *et al.*, 2007).

Treg cells achieve suppression by multiple mechanisms involving specific cytokines and other factors, but the principal processes are via bystander suppression and so-called infectious tolerance (Fig. 2) (Jonuleit *et al.*, 2002). In bystander suppression, antigen-activated Treg cells express cytokines such as IL-10, transforming growth factor (TGF)-β, and IL-35 that suppress local effector T cells irrespective of antigen specificity (Fig. 2) (Masteller *et al.*, 2005; Babu *et al.*, 2006; Walsh *et al.*, 2009). In contrast, during infectious tolerance, activated Treg cells condition the host to promote further Treg cell populations of broader specificity (Shevach *et al.*, 1998; Cobbold *et al.*, 2009; Miao *et al.*, 2009). Infectious tolerance can be adoptively transferred between animals and can persist beyond the lifespan of the original clone. Infectious tolerance is therefore of intense interest in the context of transplantation and for cell therapy to prevent or treat autoimmune conditions such as type 1 diabetes (Han *et al.*, 1996; Zelenika *et al.*, 2001; Waldmann *et al.*, 2006).

MSCs can induce Treg cells indirectly *via* their modulating effects on DCs as described earlier (Zhang *et al.*, 2004; Aggarwal and Pittenger, 2005; Djouad *et al.*, 2007; English *et al.*, 2008; H. Li *et al.*, 2008; Wang *et al.*, 2008). It is now clear, however, that MSCs can also directly induce Treg cells in the absence of DCs (Prevosto *et al.*, 2007). This is clearly seen

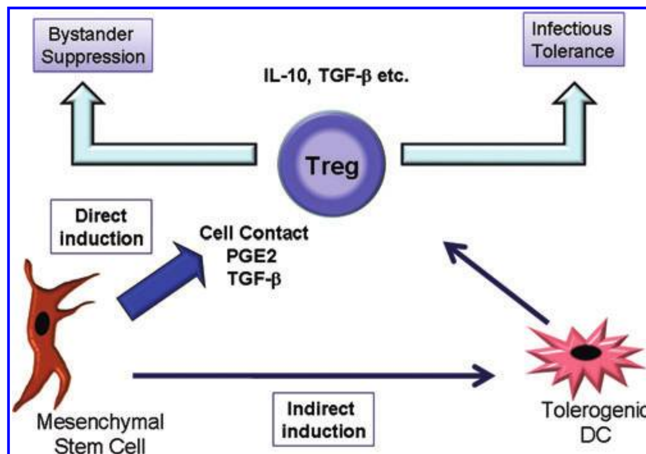

FIG. 2. Allogeneic MSCs induce a range of regulatory T-cell populations (simplified here as Treg). This can occur via an indirect route requiring tolerogenic DCs or by a direct MSC–T-cell interaction requiring cell contact and soluble factors. Once generated, Treg may suppress allogeneic rejection through multiple pathways including creation of a suppressive microenvironment (bystander suppression) or induction of further regulatory cells (infectious tolerance). The latter effect could persist well beyond the detectable presence of MSCs. Color images available online at www.liebertonline.com/hum.

when allo-MSCs are co-cultured with purified naive $CD4^+$ T cells from mice in which green fluorescent protein (GFP) has been "knocked in" to the locus for the *FOXP3* gene. Coculture with MSCs results in nuclear GFP expression (indicating activation of the *FOXP3* gene) not seen in $CD4^+$ cells cultured in the absence of allo-MSCs (L. Tobin, personal communication). A range of studies support the hypothesis that MSC-induced $CD4^+$ $CD25^{high}$ $FOXP3^+$ T cells actively suppress the immune effector responses to allo-antigen (Di Ianni *et al.*, 2008; Nasef *et al.*, 2008; Selmani *et al.*, 2008; Gonzalez *et al.*, 2009; Madec *et al.*, 2009). This is further supported by the results of human clinical studies of MSCs in the prevention of allograft rejection, GVHD, chronic inflammatory disease, and autoimmunity (Koc *et al.*, 2002; Ringden *et al.*, 2006; van Laar and Tyndall, 2006; Uccelli *et al.*, 2008; Caplan, 2009; Togel *et al.*, 2009; Tyndall and Gratwohl, 2009; Zhou *et al.*, 2010).

There are well-described roles for soluble mediators such as TGF-β1 and PGE_2 in the generation and expansion of Treg cells from $CD4^+CD25^-$ precursors (Horwitz *et al.*, 2002; Yamagiwa *et al.*, 2001; Zheng *et al.*, 2002; Baratelli *et al.*, 2005), with TGF-β1 signaling identified as a key regulator of the pathway that initiates and maintains FOXP3 expression and suppressor function (Fu *et al.*, 2004). The exact mechanisms responsible for induction of Treg cells by allogeneic human MSCs have been studied by several groups. Cell contact, PGE_2, and TGF-β1 appear to play complementary, nonredundant roles (English *et al.*, 2009; Ghannam *et al.*, 2010). Although cell contact, PGE_2, and TGF-β1 contribute to Treg cell induction by MSCs, human MSCs also secrete the soluble MHC isoform HLA-G in an IL-10- and contact-dependent manner. Leukemia inhibitory factor and HLA-G contribute to the expansion of $CD4^+$ $CD25^{high}$ $FOXP3^+$ Treg cells (Nasef *et al.*, 2008; Selmani *et al.*, 2008), and this may also explain the observations that MSCs sustain Treg cell survival and the suppressor phenotype over time (Di Ianni *et al.*, 2008). It is important that Treg cells generated by MSC encounter have been repurified and shown to further suppress alloreactivity in the absence of the original MSCs (English *et al.*, 2009). The implications of these data are that MSC therapies against autoimmunity may cast a regulatory or immunosuppressive shadow long after the original stromal cells have declined, reminiscent of infectious tolerance, and thus suggest a mechanism whereby MSCs with a brief persistence *in vivo* may have profound long-term effects.

The biological significance of MSC induction of $CD4^+$ Treg cells has also been studied *in vivo*. Pretransplant infusion of MSCs prolongs the survival of a semi-allogeneic heart transplant through the generation of Treg cells (Casiraghi *et al.*, 2008; Popp *et al.*, 2008; Ge *et al.*, 2009). Recently MSCs were also shown to prevent autoimmune β-cell destruction and subsequent diabetes mellitus by inducing Treg cells in NOD mice (Madec *et al.*, 2009) and in a similar rat model (Boumaza *et al.*, 2009). The latter autologous study also showed that MSCs sustained Treg cell responses in the periphery, supporting studies described above. Infused MSCs and rapamycin synergize to attenuate allo-immune responses and promote cardiac allograft tolerance, a process associated with tolerogenic DC and Treg cell induction (Ge *et al.*, 2009). Finally, in a murine model of asthma, MSC therapy had a beneficial therapeutic effect that was lost when Treg cells were chemically depleted (H. Kavanagh, personal communication). Taken together, these results extend the very clear *in vitro* data to show that MSC induction of Treg cells has functional relevance *in vivo*.

MSC-mediated immunomodulation occurs by multiple redundant pathways, of which $CD4^+$ Treg cell induction is only one (English *et al.*, 2007, 2008; Ryan *et al.*, 2007). For example, MSCs also induce other regulatory T-cell populations, including $CD8^+$ regulatory cells (Prevosto *et al.*, 2007) (H. Kavanagh, personal communication). Djouad and colleagues have demonstrated that MSC induction of $CD8^+$ Treg cells was responsible for at least some immunosuppressive activities of MSCs *in vitro* (Djouad *et al.*, 2003), and these cells may amplify other suppressor mechanisms (Prevosto *et al.*, 2007). The outstanding questions with regard to MSC induction of Treg cells center around the robustness of the regulatory effect, delineation of the contribution of MSCs to bystander or infectious tolerance, the precise identity of the suppressor mechanisms such as the contact-dependent signal, and the degree to which preclinical animal models translate to human disease. Answers to these questions are imminent and may well determine the ultimate utility of MSC therapy against autoimmune and other immune-mediated diseases.

MSC Suppression of T-Cell and B-Cell Effector Responses

In addition to modifying antigen presentation by DCs and promoting the expansion of Treg cell populations, MSCs may also directly influence effector functions of the adaptive immune system, including Th differentiation programs and B-cell/plasma cell activation and antibody production. These direct interactions with the effector arms of the adaptive immune system constitute an important additional element of the therapeutic effect of MSCs in diseases involving damaging inflammation, autoimmunity, or allograft rejection.

In the case of Th differentiation, there has been some debate about the modulating influence of MSCs on the T-cell subsets induced by allogeneic, autoimmune, or other model antigens. Although there have been reports that MSCs preferentially reduce Th1 responses to favor Th2-like cytokine responses (Batten *et al.*, 2006; Li *et al.*, 2007; Wang *et al.*, 2008; Lu *et al.*, 2009), this is unlikely to be a universal feature. For instance, in the context of MSC-induced Treg cells, Th2 responses are effectively suppressed at a level sufficient to reduce pathology *in vivo* (Nemeth *et al.*, 2010; Sun *et al.*, 2010) (H. Kavanagh *et al.*, personal communication). With regard to other $CD4^+$ populations, Ghannam *et al.* (2010) showed that MSCs prevented the *in vitro* differentiation of naive $CD4^+$ T cells into Th17 cells and inhibited the production of the effector cytokines IL-17, IL-22, IFN-γ, and TNF-α by fully differentiated Th17 cells. Furthermore, under inflammatory conditions, MSCs appear to mediate the adhesion of Th17 cells via CCR6 and exert anti-inflammatory effects through the induction of a Treg phenotype in these cells (Ghannam *et al.*, 2010). These data support earlier studies showing that MSCs ameliorate experimental autoimmune encephalitis through suppression of $CD4^+$ Th17 cells (Rafei *et al.*, 2009b), again suggesting that MSC immune modulation of T-cell subsets is not merely a rebalancing of the Th1–Th2 axis. In summary, there is now a considerable body of data demonstrating that MSCs have potent direct suppressive influences on effector CD4+ T cells while promoting and sustaining Treg cells.

Studies of MSC effects on B-cell function—particularly the *in vivo* production of antibody during antigen-specific immune responses—have been less frequent and have produced some conflicting results (Corcione *et al.*, 2006; Gerdoni *et al.*, 2007; Rasmusson *et al.*, 2007; Comoli *et al.*, 2008; Rafei *et al.*, 2008; Tabera *et al.*, 2008; Traggiai *et al.*, 2008; Asari *et al.*, 2009; Schena *et al.*, 2010; Youd *et al.*, 2010). Many antibody responses are dependent on T-cell help, and so it is important to consider the distinction between direct MSC modulation of B cells and indirect B-cell effects resulting from suppression of DCs and T cells. *In vitro* experiments involving coculture of human MSCs with purified B-cell populations under a variety of stimulatory conditions have predominantly shown inhibition of B-cell proliferation (via G0/G1 cell cycle arrest), differentiation, immunoglobulin production, and chemotaxis with preserved or improved cell survival (Corcione *et al.*, 2006; Comoli *et al.*, 2008; Tabera *et al.*, 2008). Similar observations have been reported for purified mouse B cells and plasma cells *in vitro* (Rafei *et al.*, 2008; Asari *et al.*, 2009; Schena *et al.*, 2010). Mediators that have been identified for MSC suppression of B-cell functions to date include alternatively cleaved CCL2 (Rafei *et al.*, 2008), IFN-γ, and PD1/PDL1 interaction (Schena *et al.*, 2010). In contrast, however, several groups have reported stimulatory effects of MSCs on *in vitro*–activated B cells or plasma cells from healthy humans (Rasmusson *et al.*, 2007) or patients with systemic lupus erythematosis (Traggiai *et al.*, 2008). The reasons for such apparently contradictory results are not entirely clear but may include variability in the sources and properties of MSCs as well as in the different antigen-dependent and polyclonal stimuli that have been used to activate B cells in culture.

The limited numbers of studies carried out in *in vivo* models of pathogenic antibody production have also yielded inconsistent outcomes. MSC-mediated inhibition of antigen-specific antibody production (including T-cell independent antibody responses) was observed in mice by three groups (Gerdoni *et al.*, 2007; Rafei *et al.*, 2008; Asari *et al.*, 2009), whereas others have reported failure of *in vivo* MSC administration to suppress autoantibodies or increased autoantibody titers and disease activity in a mouse model of systemic lupus erythematosis (Schena *et al.*, 2010; Youd *et al.*, 2010). It is interesting that there is also some experimental evidence that pre-existing antibody responses may be suppressed by MSCs through inhibition of plasma cell antibody production (Comoli *et al.*, 2008; Rafei *et al.*, 2008). Taken together, the existing literature regarding MSC effects on B-cell and plasma cell functions suggest a complex interaction that includes both inhibitory pathways of high clinical interest as well as the potential for stimulatory effects that could limit the benefit of MSC-based therapies for some immune/inflammatory diseases.

To What Extent Are Allo-MSCs Immunoprivileged *In Vivo*?

Despite numerous clinical trials with allo-MSCs, it remains unclear to what extent these cells elicit anti-donor immune responses *in vivo* and whether efficacy is truly equivalent for allo-MSCs compared to auto-MSCs for a given therapeutic target. A key question is whether the inherent immune suppressive properties of MSCs are sufficient to overcome the potent and diverse processes of immunologic priming, effector responses, and memory that are typically engendered by allogeneic cells in a healthy individual. As reviewed in previous sections, many aspects of the mechanistic basis for MSC-mediated immune modulation have been uncovered, and at least some of these are known to be operational *in vivo.* Furthermore, several studies have indicated that donor-specific MSC infusion prior to or at the time of allogeneic organ or tissue transplantation may delay rather than hasten allograft rejection (Bartholomew *et al.*, 2002). In humans, donor MSCs have been reported to attenuate some aspects of GVHD following allogeneic hematopoietic stem cell transplantation (Lazarus *et al.*, 2005). More recent data from animal models also suggest that MSCs of allogeneic or xenogeneic source can effectively protect from death due to sepsis (Nemeth *et al.*, 2009), neuronal loss following cerebral ischemia (Ohtaki *et al.*, 2008), and neurological injury in experimental autoimmune encephalomyelitis (Zappia *et al.*, 2005; Rafei *et al.*, 2009b) in comparable fashion to auto-MSCs.

Although such preclinical and clinical studies provide evidence in favor of a therapeutic benefit of allo-MSCs, the question of whether they enjoy complete immune privilege *in vivo* remains highly relevant to the true clinical and commercial benefits of allo-MSC therapies in the long term. In the majority of potential clinical applications it is not clearly known for how long MSCs need to persist *in vivo* in order to exert their maximal beneficial effects. For conditions in which a short-lived presence of MSCs within diseased tissue is of benefit, it is, nevertheless, likely that strong immunogenicity of allo-MSCs will have a negative influence on the potency and duration of treatment effect as well as the feasibility of subsequent dosing. For clinical applications in which permanent MSC engraftment, prolonged therapeutic effect, or subsequent allogeneic organ transplantation is anticipated,

even weak *in vivo* immunogenicity may prove to be a formidable barrier to successful translation. It is important, therefore, that basic observations regarding the effectiveness of allo-MSCs in preclinical disease models be extended to define the extent and limits of their immune privileged state. Table 1 summarizes the results of several studies in which the *in vivo* immunogenicity of allo-MSCs has been specifically examined or in which the therapeutic efficacies of allo-MSCs and auto-MSCs have been directly compared in immune competent hosts. It is important that the work carried out to date has included a variety of species and administration routes. In some studies, observations regarding immunogenicity of allo-MSCs have been strengthened by re-challenging recipient animals with donor allo-antigen through strategies such as skin grafting. In others, experimental observations of allo-MSC longevity *in vivo* provide indirect evidence for or against immune-mediated rejection. Finally, in a smaller number of studies, the influence of allo-MSC number, exposure to inflammatory cytokines, or differentiation along one or more lineages on *in vivo* immunogenicity has been examined. Some significant inconsistencies remain to be resolved, but this literature (summarized in Table 1) does allow several provocative statements to be made regarding the *in vivo* immune responses to allo-MSCs:

1. The majority of studies that have carefully analyzed donor-specific responses in immune competent rodents, pigs, and non-human primates following allo-MSC administration have generated evidence of immunogenicity. Notably, donor-specific antibody was observed in all studies in which allo-antibody assays were carried out. In several studies allo-specific responses were relatively weak, whereas in others allo-MSCs proved to be strongly immunogenic and sensitizing against subsequent donor antigen exposure (Table 1).
2. Immunogenicity and therapeutic immune modulation can co-exist in vivo. In some disease models allo-MSCs and auto-MSCs were found to be of comparable efficacy despite eliciting anti-donor immune responses, whereas in others, efficacy was lower for allo-MSCs compared with auto-MSCs (Table 1). The parallel influences of immunogenicity and suppression may be especially beneficial for proposed therapies using MSCs against autoimmune conditions. In these scenarios the ability of MSCs to induce infectious tolerance may be the critical correlate of efficacy.
3. Site of administration is an important modifier of allo-MSC immunogenicity. Sites for which allo-MSCs appeared to be non-immunogenic or very weakly immunogenic included intracranial, intracerebral, intra-articular, and implanted into skin wounds (Table 1). In contrast, intravenous, intraperitoneal, subcutaneous, and intramyocardial administration were sometimes associated with detectable anti-donor immunity and sometimes active rejection in the absence of other immune suppressive therapy (Table 1).

Overall, it is reasonable to state at this time that MSCs have the capacity to initiate both cellular and humoral allo-immune responses *in vivo* but that, in some conditions, immunogenicity may be considerably attenuated compared with other allogeneic cell types because of inherent anti-inflammatory and immune modulatory properties.

Some additional important issues are linked to the basic question of the *in vivo* immunogenicity of allo-MSCs and its significance for their therapeutic application. First, it remains unclear whether allo-MSC immunogenicity is altered following differentiation into chondrocytes, osteocytes, or other lineages. This consideration is of particular relevance to the use of MSCs in bone and joint disease but has been little studied to date. Nonetheless, it has been shown in rabbits that osteogenic cells differentiated from MSCs retained immunosuppressive properties *in vitro* and functioned as osteoblasts in allogeneic hosts for up to 28 days without precipitating primary rejection or sensitizing to a subsequent MSC-donor specific skin graft (Liu *et al.*, 2006). Second, it is of interest to know whether immunosuppressive therapies currently prescribed in organ transplantation can be effectively used to prevent anti-donor immune responses to allo-MSCs *in vivo* without diminishing therapeutic efficacy. In this regard, Poncelet *et al.* (2008) have shown, in a miniature pig model of myocardial infarction, that the calcineurin inhibitor tacrolimus significantly attenuated the anti-donor antibody response to allo-MSCs delivered directly into the infarct. Recently, Ge *et al.* (2009) also demonstrated that a combination of allo-MSC infusion and low-dose sirolimus (rapamycin) therapy resulted in long-term survival of fully MHC-mismatched heart transplants in mice. As a final issue, allo-MSCs may be deployed into sites of inflammation, rich in pro-inflammatory mediators such as IL-1, TNF, and IFN-γ. The questions arise, therefore, whether allo-MSC immune modulation persists and anti-donor immune responses are enhanced in the inflamed tissue environment. Fortunately, several advances have been made in this regard. Stimulation of MSCs with IFN-γ upregulates both MHC class I and II (Le Blanc *et al.*, 2003a,b), which may render these cells susceptible to rejection in an immune competent host especially as an elevated MHC class I level makes the cells vulnerable to cytotoxic T-cell-mediated lysis *in vitro* (S. Schu *et al.*, manuscript submitted). In a mouse model of experimental autoimmune encephalomyelitis, IFN-γ increased MSC expression of CCL2, MHC I, and MHC II, leading to loss of disease suppression and allo-MSC rejection (Rafei *et al.*, 2009a). In the pig, Cho *et al.* (2008) have demonstrated that both T-cell and antibody responses to allo-MSCs were enhanced *in vivo* by pre-exposure of MSCs to IFN-γ. Thus, there is evidence that allo-MSC immunogenicity and rejection may be more of a barrier to successful therapeutic application in the setting of localized inflammation. In contrast, it is also well established that exposure of MSCs to some inflammatory signals (*e.g.*, high-dose IFN-γ) can enhance their suppressive effects on T cells, monocyte/macrophages, and DCs (English *et al.*, 2007; Ryan *et al.*, 2007; Polchert *et al.*, 2008; Opitz *et al.*, 2009). In models of GVHD, chronic obstructive pulmonary disease, and allergic airway disease, prestimulation of MSCs with IFN-γ improves the efficacy of cell therapy (Polchert *et al.*, 2008) (B.P.M., unpublished data; H. Kavanagh *et al.*, manuscript submitted). Mechanistically, these observations have been linked with IFN-γ-mediated upregulation of IL-10, TGF-β1, PGE_2, and, in particular, the immune suppressive enzyme indoleamine 2,3-dioxygenase (English *et al.*, 2007; Ryan *et al.*, 2007; Popp *et al.*, 2008; Opitz *et al.*, 2009; Crop *et al.*, 2010). Although paradoxical in some senses, the literature in this area indicates that allo-MSCs introduced into a site of existing tissue injury and inflammation engage in a

Table 1. Selected Studies Examining the *In Vivo* Immunogenicity of Allo-MSCs and/or Longevity and Efficacy in Comparison to Auto-MSCs

Disease/model	*Species*	*Route*	*Comparison with auto-MSCs?*	*Anti-donor T-cell response*	*Anti-donor antibody response*	*Response to donor antigen re-challenge*	*Longevity* in vivo	*Efficacy* in vivo	*Ref.*	*Conclusion*
Immunogenicity	Rhesus macaque	Intracranial	No	Increased circulating T cells	Weak	No lytic response to *in vitro* re-challenge	NT	NA	Isakova *et al.* (2010)	Immunogenic (weak)
Allogeneic bone marrow transplant	Mouse	i.v./i.p.	Yes	NT	NT	Accelerated rejection after allo-MSCs	Allo-MSC survival shorter than auto-MSC	Allo-MSCs equal to auto-MSCs	Zangi *et al.* (2009)	Immunogenic: allo-MSCs therapeutic but immunogenic in immune competent hosts
Immunogenicity	Rat	Intracerebral	No	NT	NT	NT	Allo-MSCs persist > 60 days	NA	Rossignol *et al.* (2009)	Non-immunogenic: allo-MSCs not rejected by 63 days
Skin wound healing	Mouse	Implanted into skin wound	Yes	Local T-cell numbers similar to no MSC control	NT	NT	Allo-MSC survival similar to auto-MSC	Allo-MSCs equal to auto-MSCs	Chen *et al.* (2009)	Non-immunogenic: allo-MSCs similar efficacy and survival to auto-MSCs
EAE	Mouse	i.p.	Yes	NT	NT	Increased T-cell response on re-challenge by IFN-γ-treated allo-MSCs	NT	Allo-MSCs equal to auto-MSCs	Rafei *et al.* (2009b)	Immunogenic (conditional): allo-MSCs are therapeutic but may be rejected following IFN-γ pre-treatment
Localised brain injury	Rat	Intracerebral	No	Increased local T cells with allo-MSCs	NT	NT	Allo-MSCs detectable at 24 days	Allo-MSCs ineffective	Camp *et al.* (2009)	Immunogenic (weak): allo-MSCs are immunogenic but not rejected
Sepsis (cecal puncture)	Mouse	i.v.	Yes	NT	NT	NT	NT	Allo-MSCs equal to auto-MSCs	Nemeth *et al.* (2009)	Immunogenicity not directly addressed. Allo- and auto-MSCs equally therapeutic
Kidney ischemia reperfusion injury	Rat	Intra-arterial	Yes	NT	NT	NT	NT	Allo-MSCs less effective than auto-MSCs	Togel *et al.* (2009)	Immunogenicity not directly addressed. Allo-MSCs less effective than auto-MSCs
Myocardial infarction	Rat	Intramyocardial	Yes	NT	NT	NT	NT	Allo-MSCs equal to auto-MSCs	Imanishi *et al.* (2008)	Immunology not directly addressed. Allo- and auto-MSCs equally therapeutic
Immunogenicity	Pig	i.v. and s.c.	No	Increased with multiple or IFN-γ-treated allo-MSC injections	Detected with multiple or IFN-γ-treated allo-MSC injections	Increased with multiple or IFN-γ-treated allo-MSC injections	NT	NA	Cho *et al.* (2008)	Immunogenic (conditional): immunogenicity increased with multiple injections or exposure to inflammation

GVHD (MSCs allogeneic to host, autologous to donor)	Mouse	i.v.	No	NT	NT	NT	NT	Increased efficacy with IFN-γ-treated allo-MSCs	Polchert *et al.* (2008)	Immunology not directly addressed. IFN-γ-stimulated MSCs superior efficacy.
Immunogenicity	Mouse	i.p.	Yes	Increased CD4 T-cell response	Detected	Accelerated skin graft rejection after allo-MSCs	NT	NA	Badillo *et al.* (2007)	Immunogenic: allo-MSCs induce anti-donor T-cell and antibody responses
Myocardial infarction	Pig	Intra-cardiac and s.c.	No	Increased	Detected	Increased alloantibody with re-challenge	NT	NA	Poncelet *et al.* (2007)	Immunogenic: post-MI intra-cardiac allo-MSCs more immunogenic than s.c.
In vivo osteogenesis (allo-MSC-derived osteoblasts)	Rabbit	Adjacent to skull and i.p.	Yes	NT	NT	Skin graft rejection unchanged	Allo-MSC-derived osteoblasts survived up to 28 days	Allo-MSC-derived osteoblasts effective	Liu *et al.* (2006)	Non-immunogenic: allo-MSC-derived osteoblasts not rejected, did not sensitize to donor skin graft
Allo-bone marrow transplant	Mouse	i.v.	Yes	Detected	NT	Accelerated donor cell rejection	NT	Allo-MSCs resulted in graft rejection	Nauta *et al.* (2006b)	Immunogenic
Immunogenicity	Baboon	i.v. and i.m.	No	Decreased	Detected	Second allo-MSC dose not rejected by 4 weeks in 50%	Second allo-MSC dose survived up to 4 weeks in 50%	NA	Beggs *et al.* (2006)	Immunogenic (weak): allo-MSCs suppressed donor-specific T cells but not anti-donor antibody response
Erythropoietin secretion	Mouse	s.c.	Yes	Increased	NT	Repeat allo-MSC injections rejected more rapidly	Allo-MSC effect diminished between 20 and 40 days	Allo-MSCs less effective than auto-MSCs	Eliopoulos *et al.* (2005)	Immunogenic
Allogeneic-tumour growth	Mouse	Intra-articular and i.m.	Yes	NT	NT	NT	Bone derived from allo-MSC clone detectable at 60 days	NA	Djouad *et al.* (2003)	Non-immunogenic: allo-MSC cloned line was not rejected

EAE, experimental autoimmune encephalitis; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; NA, not applicable; NT, not tested; post-MI, post--myocardial infarction; s.c., subcutaneous.

complex, active cross-talk with the cells and mediators around them. On the one hand, these interactions may result in further induction of beneficial, immune suppressive features of the MSCs, whereas, on the other hand, they may render the MSCs more susceptible to lysis by cytotoxic T cells and NK cells. A better recognition that these two processes are not mutually exclusive and should be carefully studied in parallel in the future will be an essential step toward improving allo-MSC-based therapies to the point of routine clinical use for inflammatory and immune-mediated diseases.

Outlook

The field of MSC-related immune modulation has reached an exciting juncture. Despite some lingering controversy remaining regarding the degree to which MSCs truly differ from fibroblasts (Jorgensen, 2010), it has become very clear that stromal progenitor cells from multiple sites interact dynamically with almost every component of the immune system and, as reviewed here, do so with predominantly suppressive effects. The list of potential therapeutic applications continues to grow, and the use of pre-expanded allo-MSCs appears to be the most practical and commercially viable approach. In confronting the question of why recent large clinical trials produced disappointing results, therefore, it must be acknowledged that some assumptions made about the interaction between allo-MSCs and the host immune response *in vivo* may have been overly simplistic. Furthermore, as the specific mechanisms whereby MSCs exert their beneficial effects in a given disease remain poorly understood, there are few genuine, quantifiable, correlates of efficacy upon which to base comparisons between different sources of MSCs. From our perspective, the literature to date supports a view that therapeutic MSCs are conditionally subject to allo-immune responses *in vivo*. Furthermore, there is experimental evidence to suggest that cellular and humoral anti-donor responses in immune competent recipients are sufficient, in some settings, to limit MSC longevity, attenuate beneficial effects, and sensitize to subsequent allo-antigen exposure. It is important that the available literature also provides reasons to believe that allo-MSCs may be truly immune privileged at some anatomical sites or that detrimental anti-donor responses to allo-MSCs may be readily controlled or even converted to donor-specific immune tolerance. In the future, achieving the optimal benefits of allo-MSC therapy for each disease process will require further careful comparisons with autologous cells coupled with a more rigorous application of methods in transplant immunology to ongoing preclinical and clinical studies.

Acknowledgments

The authors are supported by a Science Foundation Ireland Centre for Science and Engineering Technology grant to the Regenerative Medicine Institute (REMEDI) (to M.D.G., T.R., and B.P.M.) and by individual grants from the Science Foundation Ireland (SFI): SFI 06/RFP/BIC056 and SFI PI 07/IN.1/B925 (to T.R.) and SFI PI 06/IN.1/B652 (to M.D.G.). Dr. H. Kavanagh and Ms. Laura Tobin are thanked for sharing unpublished data with the authors during the preparation of this article.

Author Disclosure Statement

M.G., T.R., and B.P.M each declare that no competing financial or other competing interests exist.

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Received for publication July 30, 2010;
accepted August 18, 2010.

Published online: November 8, 2010.