Treatment of Inflammatory Diseases with Mesenchymal Stem Cells

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Abstract: Human mesenchymal stem cells (hMSCs) are rare progenitor cells present in adult bone marrow that have the capacity to differentiate into a variety of tissue types, including bone, cartilage, tendon, fat, and muscle. In addition to multilineage differentiation capacity, MSCs regulate immune and inflammatory responses, providing therapeutic potential for treating diseases characterized by the presence of an inflammatory component. The availability of bone marrow and the ability to isolate and expand hMSCs *ex vivo* make these cells an attractive candidate for drug development. The low immunogenicity of these cells suggests that hMSCs can be transplanted universally without matching between donors and recipients. MSCs universality, along with the ability to manufacture and store these cells long-term, present a unique opportunity to produce an "off-the-shelf" cellular drug ready for treatment of diseases in acute settings. Accumulated animal and human data support MSC therapeutic potential for inflammatory diseases. Several phase III clinical trials for treatment of acute Graft Versus Host Disease (GVHD) and Crohn's disease are currently in progress. The current understanding of cellular and molecular targets underlying the mechanisms of MSCs action in inflammatory settings as well as clinical experience with hMSCs is summarized in this review.

Keywords: Mesenchymal stem cell, immunogenicity, immunomodulation, cellular target, mechanism of action, regeneration, inflammatory diseases, clinical trial.

I. INTRODUCTION

Mesenchymal stem cells (MSCs) can be isolated, expanded and utilized for a variety of therapeutic applications including the treatment of inflammatory, cardiovascular, and orthopedic diseases. MSCs can be isolated from many tissues throughout the body. One of the richest and most readily available sources of these cells is the bone marrow. MSCs were first discovered in bone marrow by their adherence to tissue culture plastic [1]. These cells can be isolated from the bone marrow and separated from hematopoietic stem cells through *ex vivo* expansion and serial passaging on plastic. MSCs self-renew and differentiate and therefore are identified as adult stem cells. These cells have been shown to differentiate into cells of mesodermal lineage including bone, fat, cartilage, tendon and muscle [2-4]. Evidence suggests that these cells might also be able to differentiate along the ectodermal [5, 6] and endodermal [7] lineages, but whether full differentiation down these pathways is actually achieved is still under investigation. While their ability to differentiate is important, other significant functions of these cells include regulating hematopoiesis, secreting factors that aid wound healing by preventing apoptosis and stimulating endogenous cellular repair, and controlling inflammatory and immunological reactions.

MSCs are important immunoregulatory cells in the body because they respond to inflammation by homing to affected tissues and then controlling inflammation locally at the site. An essential characteristic of MSCs is that they express a variety of chemokine and cytokine receptors and can home to sites of inflammation by migrating towards inflammatory chemokines and cytokines [8-13]. The homing ability of MSCs is important therapeutically because it allows for ease of administration. The cells can be delivered intravenously and they will home to sites of inflammation, where they will respond to the microenvironment and perform local immunoregulatory actions. MSCs carry out their immunomodulatory actions in several ways. MSCs have been shown to regulate T-cell function both *in vitro* and *in vivo* [14]. MSCs can regulate an innate immune response by signaling dendritic cells to direct an anti-inflammatory T-cell response [15] and by directly suppressing natural killer cell functions [15]. MSCs also affect an adaptive immune response by exerting their immunoregulative effects through direct interaction with T-cells [15]. These immunoregulatory effects of MSCs occur in a localized tissue environment [6, 16, 17] and not systemic. This is unlike steroid therapy where systemic suppression can lead to major clinical complications. MSCs can also promote tissue regeneration by recruiting endogenous stem cells to sites of injury as well as signal local stem cell differentiation [18-20].

As a result of the unique MSC properties of specific homing to damaged tissues, regulating immune and inflammatory responses at the target sites and facilitating repair of damaged tissue, MSC infusions have therapeutic potential for the treatment of inflammatory and immune-mediated adverse reactions, such as organ transplant rejection, GVHD, allergy and autoimmune diseases. MSCs are immune privileged and as such can be delivered without Human Leukocyte Antigen (HLA) matching and the need for immunosuppression. Under appropriate conditions, they also can be expanded in culture to high numbers, and it is predicted that one bone marrow donation can yield thousands of therapeutic treatments.

The universality of MSCs, impact of manufacturing methods, immunoregulatory capabilities, and clinical evidence from early clinical studies of an allogeneic product

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manufactured under GMP conditions will be discussed in this review.

II. UNIVERSALITY OF MSCs

MSCs Low Immunogenic Profile is the Basis of Cell Universality

MSCs are naturally immune privileged cells. MSCs from children have persisted in mothers for the mother's entire life span suggesting that these cells transferred from the fetus to the mother through the placenta and were able to escape immune surveillance for almost 40 years [21]. Their immune privileged status is at least partly due to their low immunogenicity profile. Human MSCs express low levels of major histocompatability complex (MHC) class I antigens, and are negative for MHC class II, and co-stimulatory molecules CD40, CD80, and CD86 [22].

While lack of MHC class II is necessary for escaping immune surveillance, the presence of MHC class I may also be important. Low levels of MHC class I protects cells from natural killer cell mediated cytotoxicity whereas cells that do not express MHC class I are targeted and destroyed [23]. MSCs escape recognition by alloreactive natural killer cells while K562 cells (a chronic myelogenous leukemia cell line) are lysed by natural killer cells due to their lack of MHC class I expression [24]. Thus, low levels of expression of MHC class I may be advantageous for MSC therapy and transplant tolerance.

A recent study suggested that HLA-G, a non-classical MHC class I antigen, is expressed by hMSCs and may be responsible for inhibiting an immune response against MSCs [25]. Blocking the expression of this molecule caused an increase in human lymphocyte proliferation measured by mixed lymphocyte reaction containing allogeneic hMSCs [25]. The study also shows that HLA-G expression decreases as the number of cell passages increases suggesting that culture conditions could impact the immunogenicity of MSCs [25].

Immunogenicity of Animal-Derived MSCs

Even though these cells are immune privileged, there have been a few reports in the literature suggesting that these cells can elicit an immune response. For example, there have been mouse studies showing that an immune response was mounted against murine allogeneic MSCs [26, 27]. One of these reports showed that primary and memory immune responses were mounted against allogeneic C57B1/6 mouse MSCs delivered to immunocompetent Balb/c mice [26]. Another report showed that allogeneic MSCs induced a T-cell response in naïve mice following injection [27]. The immunological responses in these models are not surprising because mouse MSCs have been shown to constitutively express MHC class II molecules and costimulatory molecule (CD80) even without stimulation from interferon γ (IFN-γ) [26]. The expression of MHC class II antigens and co-stimulatory molecules on mouse MSCs is the likely reason for an immune response against donor mouse MSCs instead of tolerance induction. While MHC class II antigens are expressed on mouse MSCs, they are not expressed on rat or species higher on the evolutionary scale such as canine, swine, baboon, and human.

A study recently conducted in pigs showed that swine injected with allogeneic MSCs developed cellular and humoral responses specifically against the donor MSCs with antibody-complement-mediated cytotoxicity [28]. Although the mechanisms of immune response against allogeneic MSCs were not addressed by the authors, *ex vivo* cell culture conditions, which are varied between different laboratories, can make MSCs immunogenic if not appropriately controlled. Several reports have suggested that culture conditions can have a profound effect on MSC immunogenicity [29-31]. While some animal studies have reported an immune response against donor MSCs, an immune response has not been seen in humans. Recent reports demonstrate the absence of anti-allogeneic MSC antibody formation and absence of T-cell sensitization in patients exposed to allogeneic human MSCs [31, 32].

Effects of Ex Vivo Culturing on MSC Immunogenicity

Ex vivo culture conditions utilized by different laboratories may contribute to the varying reports of immune responses against MSCs. Of particular interest is the treatment of animal-derived reagents often used in the cell culture process, fetal bovine serum (FBS), which contains bovine serum albumin (BSA), and porcine trypsin. Porcine trypsin is used to cleave cell-to-cell and cell-to-surface matrix adherence bonds, generating single cell suspensions. Both proteins are known allergens, which can lead to potential adverse reactions in patients susceptible to bovine and porcine products, and cause non-allergic patient sensitization leading to allergic reactions upon multiple exposures at certain doses [33-36].

In addition to BSA, another potent immunogen, bovine apolipoprotein B-100 (apoB-100), has been identified in FBS-cultured cells. A recent report showed that this xenoantigen elicits an immune response in mice and humans. The authors show that FVB/N mice will produce xenoantibodies primarily against apoB-100 following administration of BL6.9 mouse embryonic stem cells cultured in the presence of FBS. The report also shows that humans will produce xenoantibodies specific for apoB-100 against autologous T-cells that have been cultured in FBS [29].

Another study in human subjects detected alloantibodies against FBS proteins in patients who were administered donor hMSCs cultured in FBS [31]. In contrast, no alloantibodies against the donor allogeneic MSCs were found. In a third report different culture methods were used to change the amount of FBS contaminants in cellular products, and the immune reaction in recipients was directly related to the level of residual FBS proteins. These investigators replaced FBS with species-specific serum for the last 48 hours of culture thereby removing up to 99.9% of FBS contaminants [30]. MSCs grown under the resulting culture conditions were tested for immunogenicity in a rat model. Syngeneic rat MSCs were grown in the presence of FBS and infused into Sprague-Dawley rats. In one experimental group, MSCs were cultured in adult rat serum supplemented with epidermal and basic fibroblast growth factors for the last 48 hours. This procedure eliminated xenoantibody formation against FBS proteins, as shown by ELISA and immunoblot analysis. The theory was that internalized FBS proteins were removed from the cells during culture [30]. Thus, accumulated data

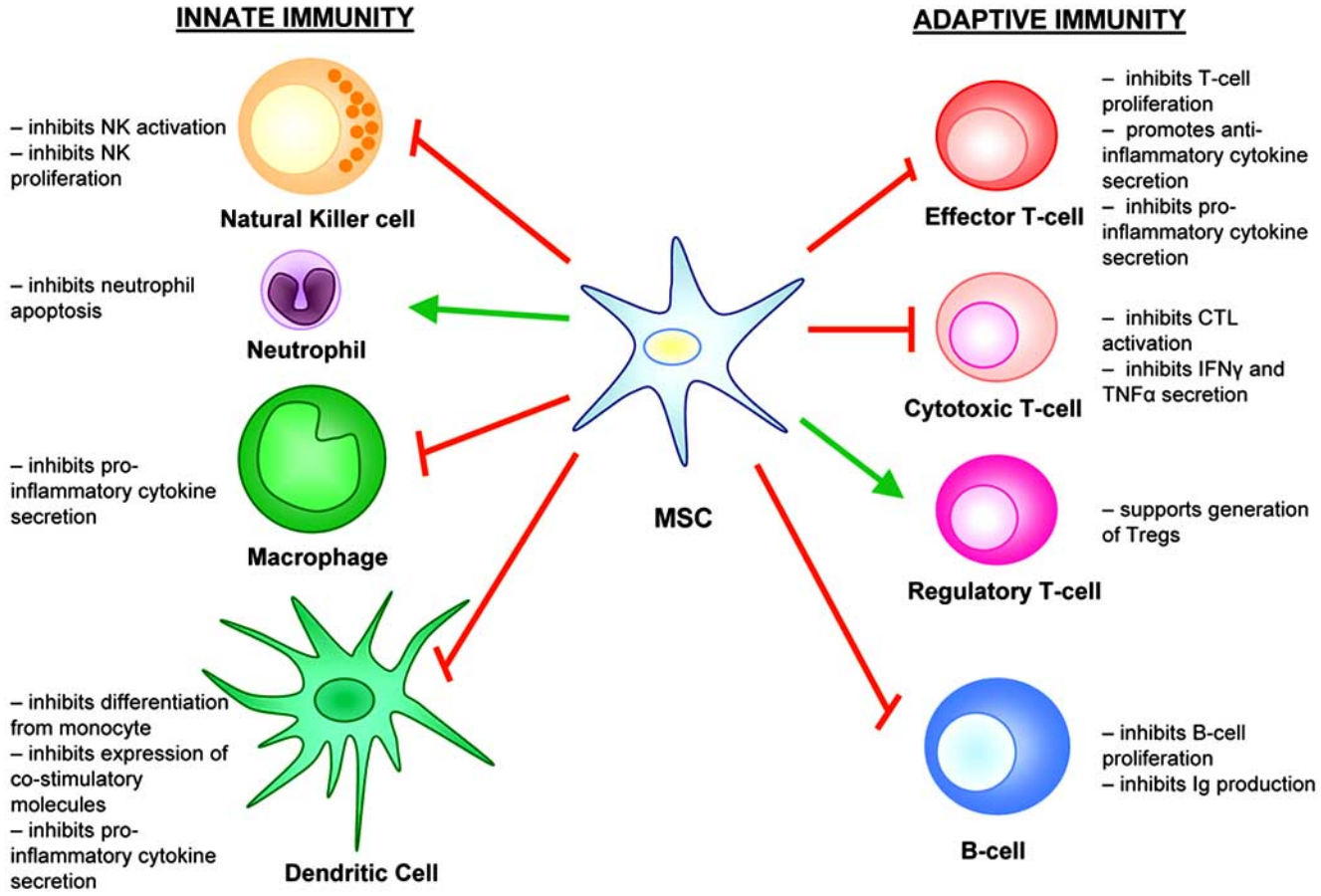

Fig. (1). Cellular Targets of MSCs.

suggest that MSC immunogenicity could be affected by the culture conditions during *ex vivo* cell manufacturing.

III. IMMUNOREGULATION BY MSCs: CELLULAR TARGETS AND MOLECULAR MECHANISMS

An important function of MSCs is their role as potent immunomodulators. This was first observed when MSCs were shown to inhibit T-cell proliferation both *in vitro* and *in vivo* [14, 37]. Subsequently, further studies have demonstrated that MSCs are able to regulate the immune system through cells of both the innate and adaptive immune systems (Fig. **1**).

MSC mechanisms of action involve both soluble factors (Table **1**) [22, 37] and direct intracellular contacts (Table **2**) [38]. Data presented below do not separately discuss results obtained using human versus mouse MSCs, however due to species-specific differences between immune cells and MSCs in humans and mice it is expected that the same factors or receptors may play different roles. In some cases contradictory results were reported by different research groups for human MSCs. One possible explanation for such discrepancies is the difference in experimental models used in the studies (e.g., source, subsets and status of immune cells, type of immune cell stimuli, ratios between MSCs and immune cells, etc.) [39, 40] which actually point to the diversity of MSC-mediated immunomodulative effects rather than to contradictions.

MSCs and T-Cells: Subsets of T-Cells Targeted by MSCs

T lymphocytes are major players in the adaptive immune system. Once activated by T-cell receptor engagement, T-cells proliferate, release inflammatory cytokines and chemokines and destroy allogeneic or pathogenic stimuli. MSCs can regulate the immune response by modulating cytotoxic or helper T-cell (Th1 or Th2) activity through modulating the release of various cytokines from effector T-cells and promoting an anti-inflammatory environment. As an example, the addition of MSCs to differentiated effector T-cells led to a decrease in the release of the pro-inflammatory cytokine, IFN-γ, from Th1 cells with a concomitant increase in the release of interleukin-4 (IL-4) from Th2 cells, which has anti-inflammatory activities in Th1 mediated diseases [15]. However, in a murine model of ovalbumin-induced asthma, a Th2-mediated inflammatory disease, an MSC infusion attenuated airway hyper-responsiveness, reduced the number of eosinophils in broncheoaveolar lavage fluids and significantly decreased the release of Th2 cytokines [70]. These findings suggest that MSCs regulate T-cell immune responses dynamically.

Another effector T-cell target of MSC immunomodulation is cytotoxic T-cells (CTLs). Cells infected with viruses as well as allogeneic cells are targets for cytolytic attack by activated CD8+ T-cells. MSCs have been shown to inhibit the activation of naïve CTLs resulting in decreased lysis of allogeneic cells. However, activated CTLs are not inhibited

Table 1. MSC-Derived Immunoregulatory Soluble Factors

MSC Effects	MSC-Derived Soluble Factors	References
Inhibition of T-cell proliferation, cytokine secretion and cytotoxicity	TGF-β	[37, 41]
	HGF	[37]
	PGE_2	[15, 40, 42-44]
	IDO	[43, 45-47]
	HO-1 + NO	[48]
	LIF	[49]
	IL-6	[42, 50]
	NO	[44, 51]
	IL-1RA	[52]
	IGF	[53]
	HLA-G5/ other HLA-Gs	[49, 54]
Stimulation of T-cell proliferation	IL-6	[55]
Support of CD8+ CTL responses to viruses	IFN-γ	[56]
Apoptosis of activated T-cells	IDO	[47]
Generation of CD4+CD25+FOXP3+ Tregs	HLA-G5	[54]
	CCL1 (I-309)	[57]
	LIF	[49]
Support of IgG secretion by B-cells	IL-6	[58]
Inhibition of NK cell proliferation, cytokine secretion and cytotoxicity	TGF-β	[59]
	IDO	[45, 60]
	PGE_2	[45, 59, 60]
	HLA-G5	[54]
Inhibition of DC maturation	IL-6	[42, 50]
	M-CSF	[50, 61]
Neutrophil protection from apoptosis	IL-6	[62]
Inhibition of TNF-α release by activated macrophages	IL-1RA	[52]

TGF-β: Transforming growth factor β; HGF: Hepatocyte growth factor; PGE_2: Prostoglandin E2; IDO: Indoleamine 2, 3 dioxygenase; HO-1: Heme oxygenase-1; NO: Nitric Oxide; LIF: Leukemia inhibitory factor; IL-6: Interleukin-6; IL-1RA: Interleukin-1 receptor antagonist; IGF: Insulin-like growth factor; HLA: Human leukocyte antigen; M-CSF: Macrophage-Colony Stimulating Factor.

in the presence of MSCs and are able to lyse allogeneic cells. Interestingly, allogeneic MSCs are not targets of CTL attack [24]. MSCs may be protected from CTL attack due to the fact that MSCs inhibit IFN-γ and TNF-α (tumor necrosis factor-α) production from CTLs [71].

Regulatory T-cells (Treg) are important players in modulating immune response in that they are anti-inflammatory in nature and function to prevent hyper-immune responses. Accordingly, MSCs can exert immunoregulatory functions by inducing the recruitment and generation of Tregs. Specifically, MSCs cultured with stimulated peripheral blood mononuclear cells (PBMCs) result in generation of CD4+CD25+highFoxP3+ Tregs [15, 72]. Upon IFN-γ stimulation, MSCs secrete the chemokine, CCL1 (I-309). It is the interaction of CCL1 with its receptor, CCR8, on T-cells, that has been shown to partially mediate MSC-induced Treg generation [57]. Another mediator of Treg generation produced by MSCs is HLA-G5, a soluble form of non-classical HLA class I molecules [54]. HLA-G5 is secreted by MSCs upon intercellular contacts between MSCs and activated allostimulated T-cells and is required for Treg cell expansion in lymphocyte-MSC co-cultures [25, 54]. Receptors that mediate the intercellular contact between MSCs and T-cells remain to be identified, however adhesion receptors on MSCs and membrane bound HLA-G are potential candidates. Furthermore, Prevosto *et al.* demonstrated that MSCs contact T-cells through CD58/CD2 and CD52/CD11a and generate a FOXP3 negative CD4+ and CD8+ Treg population. These Tregs can inhibit T-cell proliferation more potently (100-fold stronger) than conventional CD4+CD25+highFOXP3 Tregs [65]. Tregs play a key role in inducing immune tolerance, and induction of immune tolerance is integral for successful treatment of inflammatory immune diseases and allogeneic cell, tissue and organ transplantation. Preliminary data observed in animal models of autoimmune diseases support the role of MSCs in immune tolerance [73, 74]. The ability of

Table 2. Immunoregulatory MSC Receptors

MSC Effects	MSC Receptors	Receptor-Partners on Immune Cells	References
Inhibition of T- cell proliferation	HLA-G	ILT2	[54]
	Jagged-1	Notch	[63]
	PD-1 pathway (PD-1, PD-L1 & PD-L2)	PD-1, PD-L1, PD-L2	[64]
Induction of FOXP3-negative CD4+ and CD8+ Tregs	CD58	CD2	[65]
	CD54	CD11a	[65]
Antigen presentation to CD4 cells	MHC class II	TCR, CD4	[66]
	VCAM-1	α4-integrin (CD49d)	[67]
Antigen presentation to CD8 cells	MHC class I	TCR, CD8	[68]
Stimulation of T-cell proliferation	MHC class II	TCR, CD4	[69]
Support of IgG secretion by B-cells	ICAM-1	CD11a	[58]

PD: Programmed death; PD-L: Programmed Death Ligand; ILT: Ig like transcript; TCR: T-cell Receptor; MHC: Major Histocompatibility Complex; VCAM: Vascular Cellular Adhesion Molecule; ICAM: Intracellular Adhesion Molecule, CD54.

MSCs to generate Tregs supports their use for treatment of inflammatory immune diseases.

MSCs have also been shown to inhibit the response of naïve and memory T-cells to their cognate antigens. The inhibitory effect of MSCs on naïve and memory T-cell response was demonstrated in studies where MSCs cultured with either naïve or memory T-cells and stimulated with its cognate antigen abrogated antigen-specific lymphocyte proliferation, cytotoxic activity of CD8+ CTLs and generation of IFN-γ producing CD8+ T-cells [38]. It was further shown that the mechanism underlying MSCs' inhibitory effect depended on the dose of MSCs and mediated by intercellular contact.

Effects of MSCs on T-Cells

The effect of MSCs on T-cell proliferation has been extensively documented both *in vitro* [14, 15, 22, 37] and *in vivo* [74, 75]. A number of studies have demonstrated that MSC immunosuppressive activity is not mediated through induction of cell apoptosis [22, 37], but rather by arresting T-cells in the G0/G1 phase of the cell cycle [76]. Markers of cell cycle Ki67 and cyclin D2 in T-cells is inhibited whereas p27kip1 expression is up-regulated in the presence of MSCs [59, 70]. MSCs support survival of unstimulated T-cells [22, 77], however MSC-induced apoptosis of activated T-cells has been reported [47]. Thus, depending on the cell status, MSCs may protect quiescent T-cells from death, arrest T-cells in G0/G1 phase of the cell cycle or promote apoptosis of activated T-cells. It has been shown that protection of T-cells from apoptosis by MSCs involves down-regulation of FAS and FAS ligand expression and inhibition of endogenous apoptotic proteases [77]. However, MSC-derived anti-apoptotic factors and/or receptors remain unknown at the present time. IFN-γ induced secretion of IDO has been linked to MSC-induced T-cell arrest and apoptosis [45, 47].

Although evidence is widely available describing the inhibitory role of MSCs on T-cell function, MSCs have also been shown to stimulate T-cells under varying experimental conditions. In one study, at low MSC to immune cell ratios (1:40-1:100), MSCs have been shown to increase proliferation of allo-stimulated T-cells by 40 to 190% as compared to control cultures without MSCs [39]. Although the underlying mechanism remains unknown, MSCs constitutively secrete low levels of cytokines (IL-1, IL-6) and chemokines (RANTES, MCP) [69] which may support the function of T and other immune cells in certain conditions [78, 79]. MSCs do not secrete IFN-γ, however data suggest that viruses may trigger IFN-γ secretion in MSCs, which may shift balance between stimulatory and inhibitory MSC-derived factors toward immune stimulation. By secretion of IFN-γ in response to viral antigens, MSCs can support expansion and cytotoxicity of CTLs [56]. In the presence of high concentrations of TNF-α mouse MSCs can support allo-stimulated T-cell proliferation by secretion of IL-6 [55], however a similar effect has not been proven for human MSCs. Culture-expanded MSCs normally do not express MHC class II antigens, they can be triggered to express MHC class II antigens by treatment with IFN-γ. However, in one study MHC class II positive human MSCs were obtained without IFN-γ stimulation. Such MHC class II expressing MSCs have been shown to increase the proliferation of unstimulated PBMCs against allogeneic MSCs and the PBMC response to recall antigens *Tetanus Toxoid*, *Bordetella Pertussis* and *Candida Albicans* [69]. Other studies have shown that IFN-γ can trigger expression of MHC class II antigens on MSCs, however in studies where MSCs are treated with IFN-γ to express MHC class II antigens no response against allogeneic MSCs was observed [32, 80]. This lack of immune cell proliferation in response to allogeneic MSCs may be explained by the secretion of suppressive factors such as IDO (indoleamine 2, 3-dioxygenase) in concert with MHC class II expression and lack of co-stimulatory molecules. Thus, MHC class II expression on MSCs without secretion of suppressive factors and/or the presence of co-stimulatory molecules [26, 27] may lead to a cell-contact-dependent increase in T-cell proliferation.

Although with limited potential, MSCs can function as antigen-presenting cells (APC) for memory immune cells. The underlying mechanisms of antigen presentation by MSCs involve membrane receptors expressed on MSCs and T-cells. MSCs pulsed with *tetanus toxoid* can stimulate pro-

liferation and cytokine production in a *tetanus toxoid*-specific T-cell line [67]. Intracellular contacts and soluble factors are essential for antigen presentation to naïve T-cells, and the minimum requirement for antigen presentation to CD4+ T-cells is MHC class II and accessory molecules. IFN-γ-treated MSCs express MHC class II and at least two accessory adhesion molecules, ICAM-2 (intracellular adhesion molecule 2) and VCAM-1 (vascular cellular adhesion molecule 1), and these signals are sufficient to promote antigen presentation to memory T-cells, such as *tetanus toxoid*-specific T-cells. Interaction between MSCs and activated *tetanus toxoid*-specific T-cells is mediated by VCAM-1 on MSCs and α4-integrin on T-cells. Pre-incubation of T-cells with antibodies against α4-integrin resulted in 80% inhibition in T-cell binding to MSCs [67]. Antigen presentation of recall antigens such as *tetanus toxoid* or *candida albicans* to memory cells by MSCs may occur only during a narrow window when concentrations of IFN-γ are sufficient to trigger MHC class II expression, but not excessive, which will have an opposite effect. Increases in IFN-γ concentration upon activation of immune cells correlates with decrease in MHC class II expression on MSCs and subsequent loss of its antigen-presenting potential [66]. MSCs can also present viral and tumor antigens to CD8+ MHC class I-restricted T-cells, however MSCs have limited ability to function as antigen presenting cells for CD8+ T-cells due to lack of LMP7, LMP10 and ERp57 expression in their antigen-processing machinery [68].

MSCs and B-Cells

B-cells are another major cell type of adaptive immunity and integral to the humoral immune response. B-cells are responsible for producing antibodies against antigens. Several reports demonstrate that MSCs can regulate B-cell functions including migration, proliferation and immunoglobulin (Ig) synthesis [58, 64, 81, 82]. *In vitro* studies have demonstrated that MSCs inhibit the proliferation of B-cells through arrest at G0/G1 phase of the cell cycle. MSCs also inhibit production of IgM, IgA and IgG by B-cells [81, 83]. However, other studies have demonstrated that MSCs can actually stimulate IgG secretion and induce proliferation of B-cells [58, 84]. Clearly, further studies are needed to determine how MSCs affect B-cells. Interestingly, in a murine model of experimental autoimmune encephalomyelitis, MSC treatment improved clinical symptoms in the diseased mice and, specifically, antigen-specific antibodies were significantly depleted as compared to untreated mice [74]. MSC effects on B-cell functions are mediated by both soluble factors and direct MSC-B-cell contacts [58, 64, 81]. In mice, the programmed death pathway-1 (PD-1), which is activated by the contact with PD-1L and PD-2L receptors expressed on lymphocytes, is partially involved in suppression of B-cell proliferation by MSCs [64]. IL-6 and the ICAM-1 receptor are proposed as potential mediators of human MSC stimulatory effects on B-cells [58]. Although the same MSC soluble factors that inhibit T-cell responses may play a role in suppression of B-cells, the nature of these factors as well as signaling molecules from mature B-cells, which trigger the secretion of suppressive factors by MSCs remain to be investigated.

MSCs and Dendritic Cells

Dendritic cells (DCs) are derived from monocytes and are potent antigen-presenting cells that act by internalizing, shuttling and presenting antigens to naïve T-cells, leading to T-cell activation. MSCs inhibit differentiation of monocytes to dendritic cells and down-regulate the expression of several presentation molecules (HLA-DR, CD1a), co-stimulatory molecules (CD80 and CD86) and cytokine, IL-12 [61]. MSCs affect phenotype, cytokine secretion and immunostimulatory activity of both immature and mature CD34+ and monocyte-derived DCs [50, 61]. The initial steps of DC differentiation from monocytes include down-regulation of CD14 expression and up-regulation of CD1a, CD83 and CD80. In the presence of MSCs, DC maturation was blocked, CD14 did not decrease and CD1a, CD83 and CD80 did not increase. Experiments using a trans-well system indicate that suppression of DC maturation is mediated by MSC-derived soluble factors. IL-6 and M-CSF (macrophage-colony stimulating factor) are responsible for maintenance of CD14 expression, however these factors are not involved in suppression of CD1a expression [50, 61]. Similar results were reported in a murine model where inhibition of DC maturation by MSCs was partially mediated by IL-6 [42]. Cytokines counteracting DC-specific GM-CSF (granulocyte macrophage-colony stimulating factor)/IL-4-induced CD1a expression remains unknown. Furthermore, MSCs have also been shown to inhibit TNF-α expression as well as increase IL-10 expression from stimulated dendritic cells [15]. These results indicate that MSC can induce an immunological tolerance *via* inhibition of dendritic cell functions. This statement is supported by Beyth *et al* who demonstrated that MSCs generate regulatory APCs, which secrete high amounts of IL-10 and suppress T-cells [85]. Recently, studies have demonstrated that MSCs induce mature dendritic cells to "transform" into a novel cell population with a cell surface marker profile similar to immature dendritic cells. While mature DCs induce potent T-cell proliferation, this novel cell population potently suppresses T-cell proliferation [86]. These findings suggest that MSCs can promote their immunoregulatory function through dendritic cells.

MSCs and Natural Killer Cells

Natural Killer (NK) cells are key players of the innate immune system and are important in targeting virus-infected cells and tumor cells. They act by releasing pro-inflammatory cytokines and directly destroying target cells. NK cells are activated by cytokines released by target cells or by cell surface receptors that bind to ligands expressed by target cells. Suppression of NK cell functions by MSCs include inhibition of proliferation, cytokine secretion and in some cases cytotoxicity [15, 45, 60, 87]. Inhibition of allo-activated NK proliferation in the presence of IL-2 is mediated by MSC-derived IDO activity, which is induced by IFN-γ secreted by activated NK-cells [45, 60]. In addition to IDO, HLA-G5, PGE_2 (prostaglandin E2) and TGF-β (transforming growth factor β) were identified as MSC-derived soluble factors, which suppress NK-cell proliferation and cytokine secretion [54, 59, 60]. MSCs have also been shown to inhibit lysis of target cells by NK cells through down-regulating surface receptors NKp30, NKp44, and NKG2D, all required for activation of NK cells. It should also be

noted that NK cells can also lyse MSCs, both allogeneic and autologous. IFN-γ has been shown to partially protect MSCs from NK cytolytic attack [87]. MSC effects on NK phenotype and cytotoxicity require direct intercellular contacts [59]. The nature of MSC immunoregulatory receptors that are involved in direct contacts between MSCs and NKs remains to be investigated.

MSCs and Neutrophils and Macrophages

Although data are limited, recent studies suggest that MSCs affect functions of neutrophils and macrophages [52, 62]. IL-6 was identified as a key MSC-derived factor protecting neutrophils from apoptosis [62], while IL-1RA (Interleukin 1 receptor antagonist) released by a subset of MSCs inhibits TNF-α production by activated macrophages [52].

Together these findings support the broad immunomodulatory roles of MSCs. Although most studies have been conducted *in vitro*, some *in vivo* preclinical and better yet clinical studies corroborate their potent immunoregulatory function. Clinical findings with MSCs will be discussed later in the review.

IV. DYNAMIC REGULATION OF MSC ACTIVITY BY CELLS AND FACTORS IN LOCAL MICROENVIRONMENT

In vitro, in vivo preclinical and clinical data indicate that MSCs have the potential to treat inflammatory immune-mediated diseases such as GVHD and Crohn's disease due to their unique cellular properties of specific homing to damaged tissues, inhibition of immune and inflammatory responses at the target sites, and facilitation of damaged tissue repair. Based on the accumulated data it is clear that MSC mechanism of action is significantly different from drugs that are currently used for GVHD and autoimmune disease treatment. Table **3** summarizes the features of MSCs, and then an overview of accumulated data supporting the dynamic regulation of MSC activity by cells and soluble factors at the sites of inflammation is presented. The findings that MSC activity is regulated by factors and cells in the local microenvironment support the potential benefits of MSC-based therapies.

Secretion of Anti-Inflammatory Factors by MSCs is Regulated by Pro-Inflammatory Cytokines

In a non-inflammatory environment, MSCs express low levels of COX-2 (cyclooxygenase 2), PGE_2, TGF-β, IDO and other factors, however pro-inflammatory cytokines dramatically up-regulate secretion of anti-inflammatory factors by MSCs. For example, IFN-γ up-regulates secretion of IDO, HGF (hepatocyte growth factor) and TGF-β; and TNF-α up-regulates secretion of PGE_2 by MSCs [15, 43, 45, 46, 88]. In Fig. (**2**), Aggarwal and Pittenger demonstrated that MSCs in culture produce low levels of PGE_2, however when TNF-α is added to the culture, the level of PGE_2 secretion is significantly upregulated [15]. Accumulated data support a hypothesis of dynamic MSC response to inflammatory stimuli released from activated immune cells. Activated T-cells release the pro-inflammatory cytokine TNF-α which interacts with the TNF receptors on MSCs and triggers the release of PGE_2 from MSCs. PGE_2 acts back upon activated T-cells and blocks the release of TNF-α (Fig. **3**).

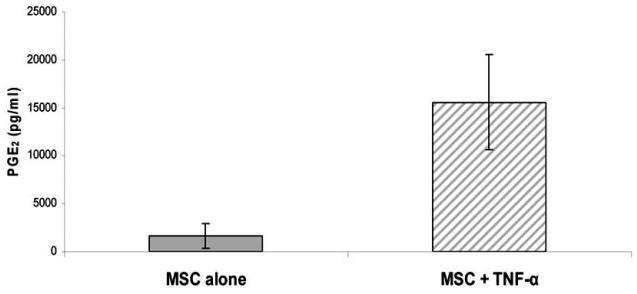

Fig. (2). TNF-α induces PGE_2 secretion by MSCs *in vitro*. Aggarwal and Pittenger performed an experiment where hMSCs were plated onto 6-well plates at 2.5 x 10^5 cells per well and incubated with or without 1 ng/ml TNF-α overnight at 37°C with 5% CO_2. Supernatants were collected and measured for PGE_2 by ELISA. Data are the average +/- SD of three experiments presented in Fig. (**4b**) in the Aggarwal and Pittenger paper [15].

MSCs Suppress Immune Response to Allo-Stimulation, But Not to Infections

With any immunosuppressive agent, the question of whether the therapy increases infection rate should be addressed. Accumulated data to date indicate that in contrast to the strong immunosuppressive effect of MSCs on allo-reactive and mitogen-induced responses, MSCs do not appear to inhibit immune cell functionality against infectious agents. Experiments have shown that in EBV and CMV-induced T-cell proliferation, IFN-γ secretion and cytolytic activity were only weakly affected by MSCs [89]. Viral infection of MSCs trigger IFN-γ secretion by MSCs, which is a key factor supporting anti-viral CTL responses [56]. MSCs also can stimulate IgG secretion by B-cells in response to bacterial LPS, CMV and VZV (Varicella zoster virus) viruses when sub-optimal levels of bacterial or viral antigens were added to cell cultures [58]. In unpurified mononuclear cell cultures, this MSC stimulatory effect on B-cells is mediated by soluble factors, however direct intercellular contacts are required for MSC-mediated stimulation of purified B-

Table 3. Therapeutic Features of hMSCs

Feature	hMSCs	Steroids, Immunosuppressive Drugs and Biologics
Biodistribution	Targeted homing to inflammation	Systemic
Inhibition of immune and inflammatory responses	Local	Systemic
Prevention and repair of tissue damage	Yes	No
Regulation of extent of immunosuppressive activity by microenvironment	Yes	No

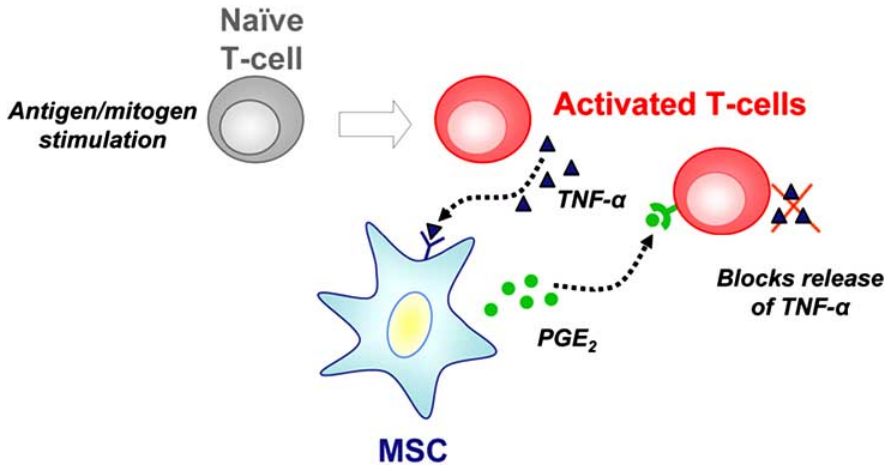

Fig. (3). Dynamic regulation of MSC activity by pro-inflammatory cytokines released from activated immune cells. Activated T-cells release the pro-inflammatory cytokine TNF-α which interacts with TNF receptors on MSCs and trigger secretion of PGE_2 from MSCs. PGE_2 acts back on activated T-cells and blocks the release of TNF-α from activated T-cells thereby inhibiting inflammatory responses.

cells. IL-6 and the ICAM-1 receptor are proposed as potential mediators of MSC stimulatory effects on B-cells [58].

In addition to MSC-derived IFN-γ for anti-viral CTL support [56], a mechanism leading to the temporary inactivation of MSC immunosuppression has been described [63]. Activation of TLR3 (Toll-like receptor 3) and TLR4 receptors by viral and bacterial-derived antigens dramatically down-regulate expression of Jagged-1 on MSCs, which has been shown to mediate MSC immunosuppressive activity *via* interaction with the Notch receptor on T-cells. Decreased Jagged-1 expression results in reversible inhibition of MSC immunosuppressive potential [63]. At the present time it is clear that TLRs play an important role not only during bacterial and viral infections, but also these receptors orchestrate the activation of the innate immune response to protozoan parasites [90]. This suggests that similar to viruses and bacteria, interaction of parasites with TLRs expressed on MSCs will result in temporary inactivation of MSC immunosuppressive activity. MSCs express a variety of TLRs including TLR2, 3 and 4 [63], which are activated by viral, bacterial and parasitic antigens. Reversible inactivation of MSC immunosuppessive activity by viral, bacterial and parasitic antigens via TLR receptors may represent a mechanism that allows immune cells to fight infections in the body.

Immune Cell Status and Ratios Between MSCs and Lymphocytes Affect MSC Immunomodulation

Investigations of molecular mechanisms underlying MSC effects on T lymphocyte functions revealed that MSCs arrest T-cells in the G0/G1 phase of the cell cycle rather than induce apoptosis [76]. MSCs support survival of unstimulated T-cells [77], however MSC-induced apoptosis of activated T-cells has been reported [47]. Thus, depending on the cell state, MSCs may protect quiescent T-cells from death, arrest T-cell division at the early steps of activation and promote apoptosis of already activated T-cells.

Numerous reports show that MSCs suppress T-cell proliferation *in vitro* induced by either antigens, mitogens or allogeneic cells [22, 37, 38]. This MSC-mediated inhibition is observed at 1:40 and lower ratios between MSCs and lymphocytes [39]. However, at MSC:lymphocyte ratios 1:40-1:100, MSC-mediated stimulation of lymphocyte proliferation was detected [39]. These results show that MSCs can both inhibit and stimulate an immune response and suggest that MSC immunomodulative activity is regulated by local microenvironment.

MSC Biodistribution is Limited to Sites of Inflammation/ Injury in the Body

It is postulated that MSCs normally migrate to sites of injury and participate in wound repair and tissue regeneration. The ability of MSCs to home to sites of acute tissue injury/inflammation has been shown in a variety of settings, including cerebral ischemia [13], total body irradiation [17] and myocardial infarction [6]. The underlying mechanism of MSC homing is a strong migratory response to specific chemotactic signals [9, 10]. The biodistribution pattern of MSCs suggests that MSC activity is limited to inflammatory sites in affected tissues.

The data presented above strongly support that MSCs activity is localized at the sites of inflammation and regulated by cells and factors present in local microenvironment. An increase in the level of pro-inflammatory cytokines in tissues will lead to an increase in secretion of anti-inflammatory factors by MSCs, while a decrease in pro-inflammatory cytokines in tissues will lead to a decrease in secretion of anti-inflammatory factors by MSCs. Such regulated tissue-specific biodistribution and activity of MSCs will help to avoid high rates of infections and other treatment-related toxicities commonly observed with the use of steroids and other immunosuppressive drugs linked to the systemic suppression of patient's immune system.

V. AUTOLOGOUS VS ALLOGENEIC MSC THERAPY

Autologous MSCs may be useful for certain therapeutic applications, however allogeneic MSC infusions have several advantages over autologous MSC infusions including their immediate "off-the-shelf" availability, and higher quality due to control of donor age and health of the bone marrow donors. Allogeneic cells can be manufactured ahead of time meaning that they are an "off-the- shelf" product immediately ready for use. The immediate availability of cells is important because they can be delivered to the patient as

soon as they are needed in acute settings. It takes several weeks to months to manufacture autologous cells, and in many cases patients cannot wait that long for treatment.

Another benefit of using allogeneic MSCs is that the age of the donor is controlled, and cells can be selectively derived from young donors. This is important because MSC number and functionality decrease with age [91, 92]. It has been shown using a colony forming unit-fibroblast assay (CFU-f assay) that MSCs per marrow cells decline as a person ages [91]. Stolzing *et al.* showed that not only did CFUs decline, but overall cellular "fitness" declined with age as determined by tests for oxidative damage, reactive oxygen species (ROS) levels, and p21 and p53 [92]. These results suggest that autologous cells from older patients will not be as effective in treating the disease as those from younger patients.

Another problem with using autologous MSCs to treat a patient is that their MSCs might have contributed to their underlying disease. For example, MSCs from patients with multiple myeloma have been shown to be functionally defective and possibly contribute to the pathogenesis of the disease [93, 94]. In addition, patients with acute myeloid leukemia have been shown to have a functional defect in their stromal layers, and there is evidence that their stromal cells may be malignant [95]. Therefore, it would not be advantageous to treat a patient with an underlying disease such as these with their own MSCs (for GVHD following a bone marrow transplant, for example) for fear that an underlying disease-causing factor might be reintroduced to these patients.

Defective MSCs may also play a role in autoimmune diseases [96, 97]. It is possible that endogenous MSCs in these patients are not completely functional. In addition, MSCs from patients with autoimmune disease are difficult to grow in culture and yield low cell numbers [96]. Using allogeneic MSCs as a therapeutic agent is a real prospect in that they do not have to be HLA matched to the recipient. While allogeneic MSCs have many advantages, there may be some circumstances where autologous MSCs are useful.

There are some potential disadvantages to the allogeneic MSCs for clinical use. Certain prospective recipients may have a personal preference against treatment with non-self, donor-derived cells. Another challenge of allogeneic MSCs is the significant time and resources needed for development, which slows their clinical availability. In comparison to autologous MSCs, allogeneic MSCs require considerable additional preclinical testing in the areas of toxicology and pharmacokinetics before clinical trials can begin. In addition to extensive preclinical testing, in-depth testing is required on the donor-derived clinical product before it is released for patient administration. Therapeutic allogeneic MSCs are manufactured in accordance with FDA GMP (good manufacturing practice) and are subjected to a series of lot release testing to ensure lot-to-lot comparability of manufactured cellular products. These tests include screening for chromosomal aberrations, viral contamination, sterility, identity, purity, and cell potency. While time and cost for product development are disadvantages, the extensive testing required for generating donor derived cells for clinical use contributes to the safety and efficacy profile of the cellular therapy.

VI. HUMAN MSC CLINICAL TRIALS FOR INFLAMMATORY DISEASES

To date, there are over 60 MSC clinical trials registered and ongoing according to the clinicaltrials.gov website in areas including inflammatory disease, cardiovascular disease, orthopedics, and organ transplant. A majority of these trials are phase 1 and phase 2 trials. The results from most of these trials are not yet available. Available data from clinical trials in the area of inflammatory disease will be the focus of the discussion below.

MSCs possess general immunomodulatory capabilities that may be used to treat a variety of disorders with inflammatory components. Osiris Therapeutics, Inc. conducted a phase 1 trial examining whether hMSCs could be safely infused and whether they could aid hematopoietic stem cell (HSC) engraftment. The infusions were well tolerated and there were no drug related serious adverse events. Over the 2-year study, a reduction in mortality from 45% to 22% was observed [98]. In 2004, LeBlanc *et al* showed that third party haploidentical MSCs successfully treated refractory severe acute GVHD in a 9-year old boy who had received a blood stem cell transplant from an HLA identical unrelated donor [32]. These data suggested that hMSCs might reduce the severe inflammation in GVHD by performing immunomodulatory functions.

In 2005, a report on a 46 patient open-label multicenter trial was published in which culture-expanded MSCs were co-administered with HLA-identical sibling matched HSCs to hematological malignancy patients. Results showed a 2-year survival rate of 53% [99]. In another clinical trial, 8 patients with steroid refractory acute GVHD grades III-IV were treated with allogeneic MSCs. Complete response was achieved in 6 out of 8 patients. Five patients were still alive between 2 months and 3 years after MSC administration, a significant improvement over the 16 control patients who were not treated with MSCs during the same time period ($P = 0.03$) [100]. Another clinical trial was conducted where allogeneic MSCs were administered to 14 children who were co-transplanted with HLA-disparate CD34(+) cells from a relative. All patients showed durable hematopoietic engraftment (an improvement over the historical graft failure rate of 15%) without any adverse reactions. The authors suggested that MSCs had modulated alloreactive lymphocytes that had escaped the preparative regimen thereby reducing the risk of graft failure [101]. Results were recently reported from a clinical trial where 55 patients with steroid-refractory acute GVHD were treated with allogeneic MSCs. Of the 55 patients, 30 achieved a complete response. Of the responders, the 2-year survival rate was 53% for complete responders compared to 16% for patients with partial or no response [102].

Osiris Therapeutics, Inc. has completed seven clinical trials to date using Prochymal® (*ex vivo* cultured adult hMSCs) to treat indications containing inflammatory components (see Table **4**). In an open-label trial studying treatment of newly diagnosed GVHD, 94% (29 out of 31) of evaluable patients responded to Prochymal® with a reduction in acute GVHD (partial and complete response) and 77% of the patients had a complete response (complete resolution of

Table 4. Completed Osiris Therapeutics, Inc. Clinical Trials

Stage	Indication	Number of Patients
Phase 2	Newly Diagnosed Acute GvHD	32
Phase 2	Pediatric Treatment-Refractory Acute GvHD	12
Phase 2	Treatment-Refractory Crohn's Disease	10
Phase 1/2	Osteoarthritis	55
Phase 1/2	Hematopoietic Stem Cell Engraftment	8
Phase 1	Hematopoietic Stem Cell Engraftment	46
Phase 1	Acute Myocardial Infarction	53

disease) by day 28 indicating a durable response to treatment (interim results reported in Kebriaei *et al*, 2008[1]). At six months, 61% of the patients treated with Prochymal® still had a durable response requiring no additional immunosuppressive therapy, clinical intervention, or increased steroid use. Previously published data indicate that less than 35% of patients achieve this endpoint when treated with steroids alone [103]. Another noteworthy result is that 95% of the patients achieving a durable response at 28 days were alive at six months. This compared favorably to patients receiving additional immunosuppressive therapy where survival was only 25%. There were no serious adverse events attributed to Prochymal® through the six month evaluation period.

In an open-label pediatric study for treatment-refractory acute GVHD, Osiris data showed that 12 out of 12 patients responded to therapy with 7 achieving a complete response and 5 achieving a partial response[2]. The 100 day survival was 58% which correlated directly with the complete response rate.

In an open-label phase 2 trial testing Prochymal® for treatment-resistant Crohn's disease, every patient treated had a reduction in disease severity by day 28. In patients who failed available drugs for Crohn's disease, there was a statistically significant reduction in the mean Crohn's Disease Activity Index (CDAI) score of 105 points by day 28. The improvement was rapid with an average CDAI reduction of 62 points by day 7. There appeared to be a positive correlation between dose and response, with patients receiving the high dose achieving a greater response (average CDAI reduction of 137 *vs* 65). In this difficult to treat population that had failed previous therapies, one-third of the patients achieved clinical remission of their disease (summary in Weiss *et al.*, 2008 [70]). Results from the phase 2 clinical trials for both Crohn's disease and acute GVHD led to the initiation of Phase 3 trials, which are currently ongoing.

In addition to acute GVHD and Crohn's disease, a phase 1 trial to treat myocardial infarction and a phase 1/2 trial for joint repair, in which hMSCs were injected directly into the knee, have been conducted (Table **4**). Both of these indications have inflammatory components that contribute to the pathology and progression of the diseases. The phase 1 cardiac trial was a 53-patient double-blind placebo-controlled safety trial where no serious adverse events were attributed to hMSCs, with trends towards improvement in function such as left ventricular ejection fraction. The Phase 1/2 trial for knee joint repair was also a double-blind placebo-controlled trial where no serious adverse events were attributed to hMSCs. Patients receiving MSCs experienced a statistically significant reduction in pain.

VII. CONCLUSIONS

In vitro, in vivo animal and human clinical data show a wide potential of MSCs for treatment of inflammatory diseases. The therapeutic potential is attributed to unique MSC properties of specific homing to damaged tissues, inhibiting immune and inflammatory responses at the target sites, and facilitating repair of the damaged tissues.

Data collected from clinical trials completed to date support the hypothesis that MSCs can perform immunomodulatory functions to suppress an adverse immunological response. Even though MSCs possess immunosuppressive capabilities, there are no evidence of immunosuppressive toxicity on a global level, systemically throughout the body, suggesting that MSCs restrict their immunomodulatory functions to areas where inflammation is present. It is likely that infused MSCs home along cytokine gradients in inflamed areas where they suppress inflammation in local microenvironments. Localized immunosuppression is much more advantageous for a patient than global immunosuppression because global immunosuppression has the severe side effect of increasing the patient's risk of infection. Therapeutic hMSCs may significantly reduce that risk. If proven to be efficacious, hMSC treatment may be highly advantageous over current anti-inflammatory therapies that are globally immunosuppressive.

Data from clinical trials suggest that hMSCs is universally well tolerated. In our experience, there have been no infusional toxicities and no adverse immunological reactions against hMSCs reported. If the results of these trials support the use of hMSCs as a therapy, then it is possible that many other severe inflammatory diseases could be treated as well.

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CONFLICT OF INTEREST

R.N., D.Y., M.L. and A.D are currently employed at Osiris Therapeutics, Inc. which is developing cellular therapeutics based on human mesenchymal stem cells.

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