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REVIEW ARTICLE

New Strategies for Overcoming Limitations of Mesenchymal Stem Cell-Based Immune Modulation

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Mesenchymal stem cells (MSCs) have rapidly been applied in a broad field of immune-mediated disorders since the first successful clinical use of MSCs for treatment of graft-versus-host disease. Despite the lack of supporting data, expectations that MSCs could potentially treat most inflammatory conditions led to rushed application and development of commercialized products. Today, both pre-clinical and clinical studies present mixed results for MSC therapy and the discrepancy between expected and actual efficacy of MSCs in various diseases has evoked a sense of discouragement. Therefore, we believe that MSC therapy may now be at a critical milestone for re-evaluation and re-consideration. In this review, we summarize the current status of MSC-based clinical trials and focus on the discrepancy between expected and actual outcome of MSC therapy from bench to bedside. Importantly, we discuss the underlying limitations of MSCs and suggest a new guideline for MSC therapy in hopes of improving their therapeutic efficacy.

Keywords: Mesenchymal stem cells, Immune modulation, Clinical trial, Limitation

Introduction

Mesenchymal stem cells (MSCs) are self-renewing multipotent progenitor cells with multi-lineage potential to differentiate into other cell types of mesodermal origin. The International Society for Cellular Therapy established the minimal criteria for MSCs, which defines MSCs as plastic-adherent cells that express specific cell-surface molecules (CD105+, CD73+, CD90+, CD11b-, CD79a-, CD19 and human leukocyte antigen (HLA)-DR-) and have the ability to differentiate into osteoblasts, adipocytes, and chondroblasts under standard *in vitro* conditions (1). MSCs have created growing interest in various fields of medicine due to their unique properties including differentiation and regenerative potential, immune modulation and migration toward sites of inflammation (2). While MSCs were initially used for tissue repair and regenerative medicine, discovery of immune-modulating mechanisms of MSCs have prompted their use in immune disorders. Currently, the therapeutic potential of MSCs has been investigated in numerous immune-mediated conditions in both pre-clinical and clinical studies, including graft-versus-host disease (GVHD), cardiovascular diseases, and chronic inflammatory autoimmune diseases.

Contrary to initial expectations, however, MSCs have failed to demonstrate clear efficacy in recent trials. Therefore, a critical evaluation of MSC therapy is needed at this point in research and development. In this review,

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we highlight the immunomodulatory properties of MSCs that contribute to their therapeutic potential. We summarize the current status of MSC-based clinical trials and focus on the discrepancy between expected and actual outcomes of MSCs from bench to bedside. Finally, we discuss the underlying limitations of MSCs and suggest a new guideline for MSC therapy to improve their therapeutic efficacy.

Immunomodulatory properties of MSCs

The rationale of MSCs as a novel therapeutic approach in a wide variety of disorders is based on their potent immunosuppressive and anti-inflammatory effects. MSCs interact with various lymphocytes and play a regulatory role in both the innate and adaptive immune system. MSC-based immune modulation primarily occurs through paracrine effects by production of soluble factors, including transforming growth factor-β (3-5), hepatocyte growth factor (HGF) (6), nitric oxide (NO) (7), hemoxygenase (HO) (8), interleukin (IL)-6 (9-11), prostaglandin E2 (PGE2) (5, 12-16) and indoleamine 2, 3-dioxygenase (IDO) (15, 17), but may also occur through direct cell-to cell contact (4, 16, 18, 19). The immunomodulatory properties of MSCs are summarized in Table 1.

Within the innate immune system, MSCs are able to inhibit the activation of pro-inflammatory monocytes and macrophages (14, 20). At the same time, monocytes and macrophages may acquire anti-immunosuppressive functions, in the presence of MSCs and their soluble factors. Classical M1 macrophages, which possess pro-inflammatory functions become alternatively activated into anti-inflammatory M2 macrophages, which are characterized by high expression of interleukin (IL)-10 and low levels of tumor necrosis factor (TNF) and interferon (IFN)-γ production (21, 22). Furthermore, MSCs inhibit the differentiation of monocytes into fully mature dendritic cells (DCs) (9, 10, 12, 13, 23-25). DCs generated in the presence of MSCs are characterized by semi-mature phenotype, in which the DC maturation markers and co-stimulatory molecules are down regulated. These tolerogenic DCs produce high levels of IL-10 and have reduced ability to stimulate allogeneic T-cell proliferation in a mixed lymphocyte reaction. Also, MSCs inhibit proliferation and cytotoxicity of natural killer (NK) cells mediated mainly through PGE2 and IDO production and often requires cell to cell contact (4, 15).

In the adaptive immune system, MSCs are able to suppress T-cell proliferation through the secretion of various soluble factors (3, 6-8, 17) and can also inhibit T-cell activation through cell-to-cell contact (18). Importantly, MSCs are able to modulate the T-cell response by orchestrating the balance between the pro-inflammatory and anti-inflammatory profiles. In an environment that consists of strong inflammatory components, MSCs are able to shift the pro-inflammatory Th1 profile to an anti-inflammatory Th2 profile (26, 27). MSCs also modulate Th17 cell subsets by preventing the differentiation of naïve Th0 cells

Table 1. Immunomodulatory effects of mesenchymal stem cells

	Cell Subset	Effects	Potential mechanism
Innate immunity	Monocytes	-Induce anti-immunosuppressive functions -Inhibit differentiation to mature dendritic cells	Through IL-6, PGE2, TGF-β, HGF production
	Macrophages	-Inhibit pro-inflammatory M1 macrophages -Alternatively activate anti-inflammatory M2 macrophages	Through IL-6, PGE2, TGF-β, HGF production
	NK cells	-Inhibit proliferation -Inhibit cytotoxicity	Through TGF-β, PGE2, IDO production and direct cell-to cell contact
	Dendritic cells	-Induce semi-mature tolerogeneic DCs with reduced ability to stimulate allogeneic response	Through IL-6, PGE2, TGF-β, HGF production
Adaptive immunity	T cells	-Suppress T-cell proliferation -Modulate inflammatory profile of helper T cells -Induce regulatory T cells	Through PGE2, TGF-β, HGF, NO, HO, IDO production and direct cell-to cell contact
	B cells	-Inhibit B-cell proliferation -Inhibit plasma cell differentiation induced by allostimulation -Inhibit Ig production	Through direct cell-to cell contact and arrest of cell cycle G0/G1

DC: dendritic cell; HGF: hepatocyte growth factor; HO: hemoxygenase; IDO: indoleamine 2,3-dioxygenase; Ig: immunoglobulin; IL: interleukin; NO: nitric oxide; PGE2: prostaglandin E2; TGF-β: transforming growth factor-β.

to Th17 cells and by suppressing the production of Th17 cytokines, including IL-17 and IL-22 (16, 28). In addition, MSCs can induce the differentiation of CD4+ helper T cells (Th0) into regulatory T cells (Tregs), a unique subpopulation of T cells that are specialized in suppressing immune responses. The coculture of MSCs with peripheral blood mononuclear cells (PBMCs) induces the differentiation of Foxp3+ Tregs through PGE2 and TGF-β (5, 29). Moreover, MSCs-induced Tregs demonstrated potent inhibitory functions against alloreactive T-cell proliferation *in vitro*.

While the effects of MSCs on B cells remain contradictory, there is evidence that MSCs have close interactions with B cells (30). MSCs are able to inhibit B-cell proliferation through cell-to-cell contact and by arrest of cell cycle G0/G1 (19, 31). Furthermore, MSCs inhibit plasma cell differentiation induced by allostimulation (32) and Ig production (33). Studies have also suggested that while MSCs are able to suppress B cells activated under various stimuli, MSCs are unable to modulate naïve or memory B cells that do not require such signals (34).

Overview of MSC-based therapies in clinical practice

Along with the initial reports on *in vitro* and *in vivo* immunosuppressive functions of MSCs (35), the first successful clinical application of MSCs to treat GVHD triggered an explosive interest in numerous research groups (36). Since the first clinical use in 2004, over 480 MSC-based clinical trials worldwide are listed in the clinical trial registry of the U.S. National Institutes of Health and more than 50 publications on MSC therapy have been reported for the treatment of at least 10 different target diseases (Table 2). The validation of safety of MSCs from different donor sources in clinical trials encouraged the commercialization of MSC products. In 2005, Osiris Therapeutics, Inc developed the first off-the shelf MSC product, Prochymal, using the bone marrow of healthy donors. In 2012, Prochymal received FDA-approval in Canada and New Zealand as a first-line treatment for acute GVHD in pediatric patients. In Korea, the first FDA approval of MSC product came in 2011 when Hearticellgram-AMI developed by Pharmicell was approved for treatment of myocardial infarctions. Subsequently, in 2012 two more mesenchymal stem cell products (Cartistem developed by Medipost for knee cartilage regeneration and Cupistem developed by Anterogen for Crohn's disease) were approved by the Korean FDA (37).

Although the initial expectations for MSC-based therapies led to the wide clinical application and rapid development of commercialized products, the therapeutic efficacy have been unsatisfactory. Series of phase I/II clinical trials using MSCs produced ambiguous results and yet further large-scale randomized trials were continued. The underlying foundation of MSC-based therapy built on uncertain clinical data led to weak clinical outcomes in the randomized placebo-controlled phase III trials of MSC products reported by Osiris Therapeutics, Inc for both acute myocardial infarction (38) and steroid-refractory GVHD (39). In addition, outcomes of Korean MSC-products have yet to publish official results in peer-reviewed journals since their FDA approval.

The contradicting clinical outcomes may be a major setback to the entire MSC field. However, we believe that MSC-based therapy, now more than ever, need thorough analysis and reconsideration in the hopes of overcoming their limitations in future studies.

Discrepancy between expected and actual results of MSC efficacy from bench to bedside

Despite the considerable progress that has been made in the development of MSC therapy for various diseases, studies have produced mixed results regarding their therapeutic efficacy. Below we describe the discrepancy between expected and actual results of MSCs from both pre-clinical and clinical studies (summarized in Table 2).

Graft-versus-host disease

Graft-versus-host disease (GVHD) is a major complication associated with morbidity and mortality following allogeneic hematopoietic stem cell transplantation (HSCT) characterized by dysregulation of inflammatory cytokines and activated donor cells that attack recipient organs and tissues. In the pilot study using MSCs for GVHD, Le Blanc et al. reported the successful use of third-party MSCs to treat steroid-refractory GVHD following allogeneic stem cell transplantation (36). This initial study provided a rationale for the use of MSCs in GVHD and encouraged further pre-clinical and clinical studies of MSCs.

Unlike *in vitro* studies that have clearly demonstrated MSCs' immunosuppressive effects against allogeneic T cell responses, *in vivo* studies have been more ambiguous. Pre-clinical studies revealed that various factors, including cell dose, timing of infusion, and pre-activated state of MSCs contribute to the therapeutic effects of MSCs. While some studies have suggested that MSCs can effectively treat GVHD in a dose-dependent manner (40, 41), others have shown that therapeutic effect could not be ob-

Table 2. Discrepancy of mesenchymal stem cell therapy: expected versus actual results

Target disease	Expected results	Actual results	
		Pre-clinical	Clinical
GVHD	Immunosuppressive effects against allogeneic responses based on *in vitro studies* Clinical efficacy based on Le Blanc's study (36)	Cell-dose, timing of infusion, and the presence of IFN-γ levels affect the therapeutic efficacy	Clinical efficacy is limited to pediatric patients with steroid-refractory acute GVHD. Efficacy depending on organ-involvement is controversial.
MI	Cardiovascular regeneration and repair of damaged myocardium Immune modulation of inflammatory response following MI through paracrine factors	MSC therapy showed overall positive results MSCs do not persist following administration indicating minimal possibility for regeneration and tissue repair	MSC therapy is well tolerated and safe, with positive results during initial follow-up Efficacy do not persist at 18 months of follow-up
IBD	Immune modulation of inflammatory response present in gastrointestinal tract	Mixed results: MSCs have produced both positive negative results in IBD Most studies focus on modification of MSCs for treatment, rather than as single agent	Systemic infusion of MSCs show limited clinical efficacy compared to intralesional treatment
MS	Immune modulation that regulate effector cells involved in pathogenesis of neurodegenerative diseases	MSC therapy was only effective when used at disease onset and not during chronic phase of disease	Limiting number of clinical trials, but feasibility and safety has been reported Mixed reports on clinical efficacy: improvements and deterioration were both reported
SLE	Immune modulation of inflammatory response involved in multiple organs	Allogeneic and umbilical cord blood derived MSCs improve renal functions but not anti-double stranded DNA antibody production levels Worsening of disease in one study was also noted	Allogeneic and umbilical cord-derived MSCs show therapeutic efficacy, but
RA	Immune modulation of Th17-related inflammatory response	Mixed results: aggravation of disease regardless of timing of treatment was noted. Positive effects of MSCs at the time of CIA induction prevented incidence	Only one report on MSC therapy for RA demonstrating feasibility: 6 months follow-up show improved clinical outcome

CIA: collagen-induced arthritis; GVHD: graft-versus-host disease; IBD: inflammatory bowel disease; IFN: interferon; MI: myocardial infarction; MS: multiple sclerosis; MSC: mesenchymal stem cells; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis.

tained at any dose (42-45). In addition to cell dose, studies proposed that the timing of treatment might play a more critical role in MSC-mediated immune suppression. Regarding the timing of administration, Polchert et al. revealed that MSCs were ineffective before GVHD development or when GVHD was too severe. This study attributed the inconsistency of MSC treatment to the difference in IFN-γ levels at different time points of GVHD development (43). Th1 cytokine, IFN-γ play a pivotal role in the immunomodulatory function of MSCs. MSCs pretreated with IFN-γ could prevent GVHD even when administered at the time of transplantation. Nonetheless, several pre-clinical studies failed to exhibit therapeutic potential of MSCs regardless of various timing and dose (2, 44, 46).

With the dramatic outcome of the first GVHD patient treated with MSCs, MSC therapy has undoubtedly showed promising results for the treatment of GVHD. In Ringden's subsequent study (47), MSCs administered in patients with grades III to IV steroid-refractory GVHD showed a complete remission rate of 75%. The European Group for Blood and Marrow Transplantation obtained similar results in a multicenter phase II study using allogeneic and third-party bone marrow derived MSCs (48). Patients with steroid-refractory GVHD were treated with MSCs from various donor sources and had a significantly reduced level or transplantation-related mortality. While the small sample size and heterogeneous treatment protocols and MSC products limit the characterization of MSC efficacy, it is becoming clear that MSC therapy may be more effective in specific environments indicating that mixed results

of MSCs for GVHD may arise depending on age, organ involvement, and severity of GVHD patients. In a multicenter trial, a greater proportion of pediatric patients responded to MSCs than adults (48). Furthermore, patients with skin-involved GVHD generally had a higher response rate to MSC treatment (49-51) whereas some studies have suggested that gastrointestinal GVHD may respond better (52, 53). In addition, studies that included both acute and chronic GVHD patients, the response was higher in acute GVHD (49, 51). It is important to recognize, however, that there is generally a lack of studies on *de novo* GVHD, chronic GVHD, and GVHD prophylaxis. Majority of studies involved patients resistant to conventional steroids and failed at least their first-line of treatment (13, 25, 43, 50, 54, 55) suggesting a better outcome in these settings. In contrast, there is some evidence that MSCs may be less effective in cGVHD (31) and GVHD prophylaxis (50).

Despite these potential variables in identifying optimal clinical settings for MSC therapy, the efficacy of MSCs is still unpredictable. In the phase III industry-sponsored trial (NCT00366145) using Prochymal for the treatment of steroid-refractory GVHD, public reports announced that Prochymal failed to achieve increased complete response rate compared to placebo controls (39, 52).

Myocardial Inflammation

Despite rapid medical advancements in the cardiovascular field, inflammatory reactions that occur upon heart diseases and failures continue to be associated high morbidity and mortality rates. Initially, MSCs were used following myocardial infarction due to their regenerative and tissue repair properties. Pre-clinical studies focused on the evaluation of MSC therapy through the measure of cardiac function and effects on cardiac remodeling (56-58). However, with the discovery that only a small portion of the injected MSCs remained present in the heart (59, 60), interest shifted toward MSC-produced paracrine factors as a critical role post myocardial infarction (61, 62). Despite different reported mechanisms of actions, MSC therapy in cardiovascular diseases of animal models have showed overall positive results.

In a pilot clinical trial, patients with acute myocardial infarction received intracoronary injection of autologous MSCs compared to saline (63). The administration of MSCs showed significant clinical improvement compared to the control group. Through cardiac electromechanical mapping, Chen et al. were able to detect viable MSCs up to three months after infusion and increased cardiac functions as demonstrated by cardiac echocardiography. Since then, further clinical trials attempted to assess the safety and efficacy of MSC transplantation for the treatment of cardiovascular diseases, including myocardial infarction and chronic ischemic cardiomyopathy (64-69). Similar to GVHD trials, the heterogeneity of clinical protocols has limited the direct comparison of MSC efficacy in cardiovascular diseases.

The different administration routes by MSC therapy may especially affect the outcomes of cardiovascular diseases. The delivery methods of MSCs include percutaneous coronary intervention, intracoronary injection, intramyocardial injection, and intravenous injection. However, direct comparison between the administration routes and following clinical outcomes is not available. There is evidence, however, that the intracoronary delivery may cause microinfarction by microvascular obstruction (70) or undesired tissue differentiation, such as bone, in the myocardium (71). Interestingly, the intravenous injection of MSCs in the randomized placebo-controlled dose-escalating trial by Osiris Therapeutics, led to an improved clinical outcome compared to the placebo group (65). However, in this study, 6-months follow-up was too short to evaluate the role of MSC in the recovery of cardiac function. Similarly, patients receiving intracoronary injection of MSCs initially showed significant improvement in left ventricular ejection compared control; however this difference did not last up to 18 months (38). Therefore, while both direct and systemic infusions of MSCs are clinically feasible for cardiovascular diseases, the efficacy of MSCs remains to be elucidated due to limited follow-up.

Crohn's Disease

Crohn's disease, also referred to as inflammatory bowel's disease (IBD), is a chronic inflammatory autoimmune disease in which the immune system attacks the gastrointestinal tract. In pre-clinical studies, MSCs were hypothesized to exert immunomodulatory effects that would improve the pathogenesis of IBD. Previously, we reported for the first time that MSCs are not clinically beneficial in IBD treatment (72). Other pre-clinical studies have suggested the positive outcomes of MSCs for the treatment of IBD; however, in these studies, MSCs were not used alone, but rather genetically modified to IL-12p40 (73), coated with antibodies (74), or genetically depleted with autoimmune regulators (75). In a single study that did claim the positive effects of MSCs, improved clinical IBD score consisting of weight loss, stool consistency and stool bleeding were noted but immune modulation of inflammatory cytokines was not described (76).

In contrast to the clinical application of MSCs for other diseases, the use of MSCs for IBD treatment demonstrates

the most homogeneity in the source of MSCs and administration protocols. Majority of trials used either allogeneic (77-82) of autologous MSCs (83) derived from adipose tissue; however, the use of bone-marrow derived MSCs has also been reported (84, 85). Furthermore, majority of clinical trials used direct intralesional infusion of MSCs mixed with fibrin glue (77-84) whereas only a single study reported the intravenous injection of MSCs (85). While MSC therapy showed local healing of Crohn's fistula through intralesional infusions, the efficacy of MSC therapy in refractory IBD patients treated with systemic infusions is ambiguous. Only a small portion of patients showed clinical response six weeks post-treatment (85). The observations that intralesional treatment of MSCs mainly improved local symptoms rather than the basic pathogenesis itself support the notion that the systemic delivery of MSCs may not be efficacious indicating the important discrepancy of MSC therapy based on administration routes.

Multiple Sclerosis

In addition to Crohn's disease, MSCs for the treatment of autoimmune diseases in various animal models, such as experimental autoimmune encephalomyelitis, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) have been reported. Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system. Initially, the therapeutic potential of MSCs have been implicated in neurodegenerative diseases of the central nervous system including stroke (86), Parkinson's disease (87), and spinal cord injury (88). Although the precise mechanisms of MSCs were not understood at the time, it was hypothesized that the immunomodulatory effects of MSCs could regulate various effector cells involved in the pathogenesis of neurodegenerative diseases. In experimental autoimmune encephalomyelitis, a model for multiple sclerosis, the intravenous administration of MSCs induced tolerance to myelin oligodendrocyte glycoprotein promoting improvement in clinical symptoms associated with the reduction of demyelination and CNS infiltration by lymphocytes (89). However, MSC therapy was only effective when used at disease onset or at the peak point during inflammation; however, not during chronic phase. Therefore, MSCs may be highly dependent on the timing of administration in multiple sclerosis, as in GVHD.

Clinical studies on MSCs for multiple sclerosis are limiting. The intrathecal administrations of autologous MSCs for these patients were feasible and safe; however, the clinical results remain unclear. While clinical improvements associated with reduced expanded disability status scale were reported in some patients, others showed no improvement or worsening of diseases (90-92). However, limited studies and small sample size prevent definitive conclusions on the effects of MSC therapy for multiple sclerosis.

Systemic Lupus Erythematosus (SLE)

Next, SLE is an autoimmune inflammatory disease with multi-organ involvement including the kidney, brain, lung, and hematopoietic systems. It has been previous suggested in both mice (93) and humans (94) that MSCs derived from the diseased individuals have abnormalities in terms of phenotype, proliferation, and differentiation. The transplantation of MSCs from healthy donors, however, was able to ameliorate the disorder (93). These observations support the use of allogeneic, rather than autologous, MSCs for SLE. In murine models, allogeneic MSCs or xenogeneic human cord-blood derived MSCs for the treatment of SLE. However, the results have not been consistent. While both human and allogeneic murine MSCs were able to alleviate renal functions associated with SLE (95), other clinical symptoms including proteinuria or double-stranded DNA levels remained unchanged (19). Furthermore, allogeneic MSCs could also enhance anti-double stranded DNA antibody production levels and worsen the disease (96).

Similarly, in the clinical setting, the use of allogeneic and umbilical cord-derived MSCs have shown therapeutic potential in active SLE patients correlated with ameliorated disease activity, improved serological markers, and stabilized renal functions (93). Furthermore, MSC therapy was feasible in patients with refractory SLE (97, 98). The results of these initial clinical trials are encouraging; however, the absence of larger randomized controlled clinical trials for SLE and other autoimmune diseases need to be resolved for further analysis.

Rheumatoid Arthritis (RA)

RA is a T-cell-mediated autoimmune disease characterized by cartilage and bone destruction. Despite continued research on the role of MSCs in RA, therapeutic potential of MSCs is controversial. In our study, we observed that MSCs are ineffective for treatment of collagen-induced arthritis (CIA), a murine model for rheumatoid arthritis, promoting Th17 related cytokines and subsequently aggravating symptoms of CIA (99). In another study, the negative effects of MSCs in CIA were associated with the presence of TNF-a, which reversed the immunomodulatory properties of MSCs and worsened clin-

ical symptoms (100). Various doses were injected at the time of immunization or booster injection; however, the aggravation of clinical symptoms was similar in both conditions. In contrast, therapeutic effects have also been demonstrated (100). A single injection of MSCs at the time of CIA induction could prevent the incidence of CIA. Interestingly, MSC treatment following the establishment of CIA could also prevent the exacerbation of disease in which 7 of 10 animals treated with MSCs showed significantly lower disease score (101).

The mixed results demonstrated in pre-clinical studies have delayed the application of MSCs for RA treatment in clinical trials. The use of MSCs in RA patients had not been reported in the clinic until recently. In 2013, Wang et al. reported the safety and efficacy of umbilical cord derived MSCs in the treatment of RA (102). In the ongoing cohort, 172 patients with active RA refractory to traditional treatments were allocated into two groups, in which patients received anti-rheumatic medication with or without MSCs. MSC therapy in RA were safe and effective in controlling the refractory disease. In MSCs-treated groups, improvements of clinical manifestations correlated with decreased levels of inflammatory cytokine and increased percentages of regulatory T cells. The clinical benefits were persistent up to 6 months with a single infusion and repeated infusions could be tolerated and further enhanced efficacy. Thus, in contrast to animal models, MSCs may provide significant benefits for RA.

Limitations of MSCs-based immune modulation

Overall, previous studies described above have demonstrated the feasibility and safety of MSCs. However, the discrepancies regarding therapeutic efficacy for most diseases have dampened the initial enthusiasm and optimism for MSC therapy. The causes for these discrepancies could be countless. Recently, Galipeau presented a review article discussing the potential variables affecting MSC therapy in response to the discrepancy between the European multicenter GVHD trials and the Osiris sponsored trial. He focused mainly on the lack of standardized MSC products throughout industrial and academic centers, including donor variance, epigenetic reprogramming and senescence followed by culture expansion, immunogenicity induced during culture and cryopreservation (103). While the procedures involved in isolation, culture, expansion, and delivery of MSCs are critical, we believe that our attention must now shift toward the MSC-based immune modulation in different clinical settings and varying inflammatory environments. The source of MSCs may be critical in models such as SLE and optimal delivery route of MSC treatment may be important in cardiovascular diseases, Crohn's fistula and multiple sclerosis that involve local injection; however, ultimately, the fundamental therapeutic benefits of MSC therapy arise from their immunomodulatory properties and their capability to elicit these properties following administration.

The lessons we have learned from pre-clinical studies of MSC therapy is that MSCs are highly-dependent on the environmental inflammatory conditions. These observations highlight the inevitable limitation of MSC therapy for immune-mediated diseases. MSCs are not constitutively inhibitory, but require the "licensing" by acute inflammatory cytokines including IFN-γ, TNF, IL-α and/or IL-1β to acquire immunosuppressive effects (104). Treatment of MSCs with IFN-γ induced increased secretion of chemokine receptor ligands ICAM-1, CXCL-10, and CCL-8 (105, 106), as well as increased production of immunosuppressive IDO (107). The role of IFN-γ in MSC-mediated immune suppression was also demonstrated *in vivo* (43). When IFN-γ knock-out mice were used as effectors of GVHD, MSCs were unable to improve the survival regardless of the time of treatment. In addition to inflammatory cytokines, immunosuppressive cytokines such as TGF-β that exist in certain environments could modulate the capacities of MSCs. Interestingly, MSCs present receptors for TGF-β and depending on the inflammatory conditions, TGF-β can reverse the immunomodulatory effects of MSCs. In combination with IFN-γ and TNF, MSCs may become less immunosuppressive (108). Moreover, the activation of Toll-like receptors (TLRs) present in MSCs, such as TLR3 and TLR4 can be activated to acquire distinct immunoregulatory functions (109). Therefore the understanding of the pathological processes involved in various diseases will be critical for appropriate clinical applications of MSCs.

Different states of inflammation can result in different responses to MSC treatment, which indicates the importance of timing of MSC administration. As described in pre-clinical GVHD model (43), a narrow window exists for MSC in which adequate levels of inflammatory IFN-γ can license MSCs. This observation might partly explain the discrepancies in GVHD models. Furthermore, while Th1-related cytokines, such as IFN-γ, is dominant in GVHD, chronic inflammatory autoimmune diseases are characterized by up-regulated Th17 levels. The effects of Th17 response on MSCs are less clear as there is evidence that MSCs may promote the expansion of Th17 cells (110-112). In the presence of pro-inflammatory cytokines, such as IFN-γ and TNF-α, MSCs produce significant

levels of IL-6 in addition to TGF-β and this combination of TGF-β and IL-6 can induce polarization of naïve T cells into Th17 cells (113). On the other hand, IL-17 may be helpful in enhancing the immunosuppressive functions of MSCs, even in low IFN-γ conditions (114). Therefore, understanding the role of Th17 cytokines on MSC-based immune modulation could provide important insights on clinical application of MSCs for autoimmune diseases including IBD, multiple sclerosis, SLE, and RA.

Furthermore, inflammatory conditions change throughout the course of pathogenesis and immune response, which may affect the plasticity of MSCs following administration. Therefore, understanding the biological fate of MSCs *in vivo* may be necessary. While the *in vivo* monitoring have been previously discussed to investigate the migratory functions and differentiation potential of MSCs (115), tracking the cell-distribution and the persistence of cells *in vivo* may indirectly address the interactions of MSCs with different cytokines depending on the location and the time-course following administration.

Why we still need MSC therapy?

Therapeutic efficacy and mechanisms of MSC therapy still remains to be elucidated. Nevertheless, MSCs are still an attractive alternative for the treatment of various diseases. First, MSCs have the ability to regenerate and differentiate into different cell lineages similar to that of embryonic stem cells. However, MSCs have several advantages over embryonic stem cells in that MSCs are free of ethical issues, have low immunogenicity and no teratoma risks. Second, MSCs can be easily propagated *ex vivo* from various sources including, bone marrow, adipose tissue, and umbilical cord blood to reach clinically relevant cell doses. Third, and most importantly, MSCs possess unique immunomodulatory and migratory features that make then attractive for treating various diseases. Despite mixed clinical outcomes and lack of established data on *in vivo* efficacy, evidence continues to suggest that, when exposed in an appropriate setting, MSCs have the potential to display a potent immunomodulatory effect. In addition, the concept that MSCs can be polarized by certain stimuli provides the potential for manipulating MSC to obtain more predictable clinical effects. Therefore, the current challenges and limitations of MSCs need to be addressed through extensive investigation of MSC based immune modulatory mechanisms and continued applications in animal models and clinical trials.

A new paradigm for MSCs: enhancing MSC-based immune-modulation

The understanding that MSCs are highly responsive to environmental stimuli provides a new guideline for both exogenous and endogenous modifications of future MSC therapies. Therefore, we suggest a new paradigm for MSC-based therapies that focuses on enhancement of MSC-based immune-modulation.

Gene-modified MSCs

Sustained expression of therapeutic genes through gene modification can significantly enhance the potency of MSCs independent of external inflammatory stimuli. IL-10 transduced MSCs have been used to treat GVHD (116). While untransduced MSCs were ineffective in suppressing the development of GVHD, IL-10 transduced MSCs significantly decreased mortality rates of mice, which correlated with decreased levels of pro-inflammatory cytokines. This clinical benefit also correlated with MSCs ability to deliver IL-10 to target inflammatory sites. Gene-modified MSCs have also been reported in various experimental autoimmune models. We have previously showed that MSCs transduced with TGF-β could potently suppress CIA models compared to untransduced MSCs (99). IL-12p40 expressing MSCs could also ameliorate murine colitis compared to normal MSCs (46). Also, MSCs engineered to overexpress IL-4 could attenuate experimental autoimmune encephalomyelitis (117).

Pre-activated MSCs

However, genetically engineered MSCs have yet to be applied in the clinical setting for immune modulation. The use of genetically engineered MSC may raise critical safety issues for clinical application. In addition to genetic manipulation, the consistent secretion of anti-inflammatory cytokines may paradoxically cause pathological immune responses depending on the factors involved in disease progression. Therefore, efforts to transiently strengthen MSC-based immune modulation may be more clinically relevant.

The pre-treatment of IFN-γ has been previously discussed in GVHD (43). Furthermore, pre-treatment of MSCs with growth factors, including fibroblast growth factor, insulin-like growth factor, bone morphogenetic protein and stem cell derived factors have been reported to enhance cell survival and cytoprotective effects of MSCs exposed to hostile environments of hypoxic myocardial ischemia (118, 119). Moreover, activation of nucleotide-binding oligomerization domain 2 expressed on human um-

bilical cord-blood derived MSCs by muramyl dipeptide induces upregulation of PGE2 which significantly enhances the inhibition of mononuclear cell proliferation (120). NOD2 signaling could be useful in that it is specifically expressed on umbilical cord-blood derived MSCs rather than other cell sources.

MSC-based combination cell therapy

One of major mechanisms of MSC-based immune modulation is the induction of regulatory cell subsets including Tregs, both *in vitro* and *in vivo* (40, 121). Therefore, MSC-mediated immune modulation is interdependent on the presence of endogenous Tregs. We postulated that soluble factors secreted by MSCs can promote the induction of Treg differentiation, and in turn, the cytokines produced by Tregs can promote immunosuppressive potential of MSCs. The combination of two immunosuppressive cell types, MSCs and Tregs, could synergistically support their functions and stabilize their plasticity. Supporting this hypothesis, a study reported that MSCs and Tregs, in combination, do not impair each other's respective functions (122). In our study, we compared single cell therapy groups (MSCs or Tregs alone) with combined cell therapy group initially in an acute GVHD model (123). We observed that the combined-cell therapy approach had synergistic immunomodulatory effects in inducing long-term survival and reducing clinicopathological symptoms of GVHD which was associated with effective inhibition of both Th1 and Th17 responses compared to MSCs-treated alone. In subsequent studies, we further investigated combined-cell therapy of MSCs and Tregs in other transplantation models, including the induction of mixed chimerism following nonmyeloablative allogeneic HSCT (124), and the prevention of allogeneic skin-graft rejection (125), and observed similar results.

Identifying therapeutic windows between conventional therapies for MSC treatment

Based on reported clinical studies, MSC therapy seems to be most effective in immunosuppressant-resistant conditions, while randomized studies involving immunosuppressant-sensitive patients did not show therapeutic efficacy. Conventional immunosuppressants used to treat immune-mediated disorders involve potent inhibition of inflammatory responses. Although additive effects of MSCs and immunosuppressants may be expected in theory, the administration of MSCs during the use of immunosuppressants such as cyclosporine A (126) and dextametasone (127) has shown to disable the immunomodulatory functions of MSCs. This suggests that sufficient levels of cytokines or various paracrine factors involved in the microenvironment may be required for supporting MSCs' functions. Therefore, either the application of MSCs in immunosuppressant-resistant patients or the timely administration of MSCs during periods of immunosuppressant tapering could be considered.

Concluding remarks

In conclusion, we are at a critical milestone of MSC therapy for application in immunological diseases. The results of many animal and clinical studies have revealed the limitations of MSCs for therapeutic use, but at the same time, these studies have addressed numerous underlying mechanisms of immune modulation illuminating the possibility for overcoming the current limitations of MSC therapy. Therefore, a shift in MSC therapy toward the focus on enhancing MSC-based immune modulation may allow MSC therapy to continue to advance forward in future studies.

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Potential conflict of interest

The authors have no conflicting financial interest.

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