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Mini Review

Defining mesenchymal stromal cells responsiveness to IFNγ as a surrogate measure of suppressive potency

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Mesenchymal Stromal Cells (MSCs) are being developed as a transfusional product to treat immune and inflammatory disorders. However, results of industry-sponsored randomized clinical trials that have utilized MSC-like cells have not met primary end points of efficacy for treatment of GvHD or inflammatory bowel disease. A better understanding of MSC mechanism of action would best inform future development strategies. MSC's immunosuppressive potential is markedly augmented by licensing by the proinflammatory cytokine, IFNγ. In the present review, we analyze the effect of MSC preparation methods on MSC's responsiveness to IFNγ and immune plasticity. The concept of pre-licensing with IFNγ on MSC's immunosuppressive, engraftment and therapeutic potential is also discussed.

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Introduction

Mesenchymal Stromal Cells (MSCs) are under intensive clinical investigation for the treatment of immune disorders and regenerative medicine, as evidenced by more than 300 registered clinical trials (clinicaltrials.gov). MSCs can be isolated from heart, dental pulp, umbilical cord blood, placenta, and peripheral blood, while the best characterized are derived from bone marrow and adipose tissue. Minimal criteria required for defining “multipotent MSC populations” iused in cell therapy are: (1) adherence to tissue culture plate, (2) cell surface expression of CD73, CD90, CD105, (3) lack of expression of CD45, CD34, CD14, CD11b, CD79a, CD19, HLADR and, (4) ability to differentiate into osteoblasts, adipocytes and chondroblasts under appropriate stimuli[1)]. MSCs are present at low frequency (0.01 to 0.001 % of nucleated cells) in bone marrow. However, their robust *in vitro* proliferative potential by standard cell culture conditions facilitates their

development as a transfusional cell therapy. A large body of pre-clinical data suggest that MSCs may be particularly advantageous to treat immune disorders due to their demonstrated suppressive effects on the proliferation and function of lymphoid and myeloid cells[2]. In support of this, MSC's therapeutic utility has been well demonstrated with animal models of experimental autoimmune encephalitis, diabetes mellitus, rheumatoid arthritis, myocardial infarction, acute lung injury, retinal degeneration, acute renal failure transplant rejection, liver fibrosis, inflammatory bowel diseases and graft versus host diseases[2]. MSCs exhibit their therapeutic effect by at least six different mechanisms: (A) immunomodulation, (B) anti-apoptosis, (C) angiogenesis, (D) support of the growth and differentiation of local stem and progenitor cells, (E) anti-scarring, and (F) chemoattraction[3, 4].

A large series of early phase clinical trials have demonstrated that MSCs are safe, but proof of therapeutic benefit by randomized clinical trials is lacking[5]. A large industry sponsored multicenter phase III randomized clinical trial demonstrated that MSC therapy (Prochymal; Osiris Therapeutics, Inc., Columbia, MD, USA) failed to meet the efficacy criteria for the treatment of steroid refractory acute graft-versus-host disease (GvHD)[6]. Another randomized Phase II study by Athersys, Inc. announced that their "off-the-shelf" allogenic, MultiStem®, failed to show efficacy in patients with ulcerative colitis (http://www.athersys.com). Considering the discrepancy between encouraging pre-clinical efficacy data and negative outcomes of these clinical studies, a better understanding of MSC biology may inform development strategies. In particular, MSC responsiveness to host inflammatory cues may provide novel translational insights. IFNγ is a well-studied immunomodulatory cytokine which modulates the immunobiology of MSCs[7, 8] and the *in vivo* suppressive properties of MSCs are likely dependent on licensing with endogenous IFNγ. The word "license" was first used by Lanzavecchia to describe the activation of APCs by T helper cells and other stimuli, which subsequently can stimulate cytotoxic killer T cells[9]. The process of licensing APCs by T helper cell is crucial to activate killer T cells. Similarly, IFNγ secreted by lymphoid effector cells is necessary to "license" MSCs to execute immune suppression. In addition, for future second-generation MSC-based therapies this naturally occurring *in vivo* licensing process can be pre-empted through prelicensing or pretreatment with IFNγ before infusion therapy. Moreover, MSC's responsiveness to IFNγ and the deployed immune activated signals such as IDO (Indoleamine 2,3-dioxygenase) is amenable to analysis and may be predictive of product potency[10]. Taking this analytic approach as a surrogate of *in vivo* MSC function, it may allow for comparison of methods used in MSC preparations for cell therapy.

MSC responsiveness to IFNγ instructs T cell fate

IFNγ is a proinflammatory cytokine produced mainly by T cells and NK cells. Utilization of MSCs for immunosuppressive therapy likely responds to available IFNγ, which converts their quiescent naïve condition to a veto state. The importance of IFNγ for augmentation of the veto properties of MSCs have been demonstrated in multiple key seminal observations: (1) MSCs in quiescent state promote T cell survival[11]; (2) Anti-IFNγ receptor antibodies abrogate MSC's suppressive properties[12]; (3) IFNγ receptor knock out MSCs do not inhibit T cells[13]; and, (4) IFNγ licensing is crucial for MSCs to suppress T cell effector functions[14]. All these studies suggest that IFNγ mediated activation determines the potency of MSC's interaction with the immune effector cells. IFNγ upregulates co-inhibitory molecules PDL1 and PDL2 on MSCs which are the ligands of PD-1 on activated T cells and upon receptor-ligand interaction, cell cycle arrest ensue on T cells[15]. IFNγ also upregulates MHC Class I and Class II on MSCs which enable them to cross-present soluble exogenous antigens to T cells like Antigen Presenting Cells (APCs)[16, 17]. However MSCs differ from professional APCs such as dendritic cells not only by lacking co-stimulatory molecules B7-1(CD80) and B7-2(CD86) but also IFNγ fails to upregulate them[14]. IFNγ inducible IDO expression plays a major role in MSC's immunosuppressive properties and defines an important component of MSC immune plasticity. IDO catabolizes conversion of tryptophan to kynurenine, which is an inhibitor of T cell proliferation. Blocking IDO catabolic activity with 1-methyl tryptophan abolishes MSC's suppressive activity on T cell proliferation *in vitro*. Another tryptophan degrading enzyme, Tryptophan 2,3 dioxygenase (TDO) has homeostatic, housekeeping role predominantly in the liver and unlike IDO does not respond to immunoactive signals[18]. Human MSCs were shown to express TDO in the resting stage and IFNγ stimulation does not upregulate its expression[19, 20]. This suggests the leading role of IFNγ inducible IDO in modulating the Tryptophan catabolic pathway and subsequent immune suppression

by MSCs. Upregulation of IDO by IFNγ can be augmented with other cytokines such as TNFα which are poor inducers of IDO, by themselves alone[21]. However, TLR activators such as Lipopolysaccharides and PolyI:C have been shown to upregulate IDO through autocrine IFNβ signaling loop independent of IFNγ[20]. Similarly, MSCs with defective IFNγ Receptor 1 can still suppress T cell proliferation[22]. This suggests that other immunosuppressive mechanisms are operative in synergy with IFNγ induced effects on MSCs to regulate T cell proliferation such as: HLA-G5, Prostaglandin E2, Galectins, Insulin-like growth factor (IGF)-binding proteins, TNF-alpha stimulated gene 6 protein (TSG-6) and alternatively cleaved CCL[22]. The breadth and effectiveness of an ongoing immune response are determined by both T cell proliferation and their effector function as defined by cytokine secretion and degranulation respectively. We have demonstrated that PDL1 and PDL2 but not IDO control T cell effector functions. Thus IDO expression by MSCs targets the proliferative response of T cells while PDL1/PDL2-PD-1 interaction regulates memory T cell function[14]. These separate but synergistic functions of IDO and PDL1/PDL2 by IFNγ licensed MSCs have significant translational implication for targeting a pathologic autoimmune response.

Translational aspects of MSC's responsiveness to IFNγ

Multiple immunoregulatory molecules are produced by IFNγ licensed MSCs leading to immunosuppression. Since it appears that IFNγ licensing of MSCs is of translational significance, assessment of IFNγ-responsiveness may be linked to clinical outcome. MSC responsiveness to IFNγ is affected by key variables. These variables can be categorized as: (1) modulators of MSC physiological fitness as a transfusional cellular product, and (2) host inflammatory milieu as a source of IFNγ. The fitness of transfused MSCs is markedly influenced by cell culture, preparation and handling methods, which are used in support of clinical trials.

1)Fitness of transfused MSCs

Most transfusion products involving blood components such as erythrocytes and platelets are stocked for a finite period of time without subzero storage due to the sensitivity of these transfusional medicines to thaw-induced dysfunction. In contrast, mobilized hematopoietic stem and progenitor cells collected by a leukaphersis procedure for the purpose of an autologous peripheral blood stem cell rescue in the setting of cancer are routinely cryobanked in DMSO. These products can be retrieved from cryostorage, thawed and immediately transfused with DMSO cryoprotectant as part of cancer care. The clinical outcome of this standard procedure as measured by hematopoietic reconstitution following myeloablative therapy is predictably good as long as content of surrogate markers of stem cell content meet well defined thresholds. The success and logistic flexibility of cryostorage of personalized hematopoietic cell products provides a road map and rationale for cryobanking of non-hematopoietic cell products like MSCs in an effort to allow for large-scale deployment of a centrally manufactured pharmaceutical. Akin to what is done with cryobanked hematopoietic cells, frozen MSCs are thawed and transfused within no more than a few hours of retrieval from cryostorage. This standard operating procedure is nearly universally used in both industrial and academic clinical trials examining the use of culture expanded MSC-like cells. But MSCs and HSCs are not the same. HSCs are intrinsically anchorage independent and do not require tethering to extracellular matrices to ensure survival and proliferation, which may be a functional advantage for their recovery post thawing & transfusion in biological systems. However, MSCs are anchorage dependent cells and absence of their attachment to a substrate following harvest and transfusion may lead to *anoikis*, a form of apoptosis induction[23]. HSCs have the capacity of self-renewal and will repopulate the bone marrow and the immune system. Hence engraftment of a small fraction of normal endogenous content is enough to get amplified in the host post transfusion. In contrast, MSCs do not replicate following transfusion and analysis of autopsy tissues of MSC transfused patients demonstrates that MSCs do not form ectopic tissue[24]. In addition, HSCs home to bone marrow and engraft a feature that transfused MSCs do not possess[25]. More importantly, we have shown that thawed MSCs display impaired immunosuppressive and homing properties. Indeed, thawed MSCs undergo a molecular heat shock response by upregulating HSP70A and HSP70B proteins, which reflect a cellular response to injury and stress. Our studies also demonstrate that thawed MSCs display impaired responsiveness to IFNγ stimulation by exhibiting attenuated STAT-1 phosphorylation[26]. In a preclinical animal model of colitis, intravenously transfused cryopreserved allogeneic MSCs had no beneficial effect[27]. In support of this, we have found that thawing induces

MSC cytoskeleton disruption with marked effects on biodistribution pattern *in vivo*[28]. Thawing compromises cell viability by at least 10-15% and we had demonstrated that dead cells do not engraft and do not interfere with the engraftment of live cells. Thus thawing induces injury to live cells, which disables their intrinsic ability to mediate immune suppression and engraftment. These defects are reversible within approximately 24-48 hours if cells are allowed to recover post-thaw in culture media[28]. In support of this, a recent clinical trial has demonstrated that 100% of the patients respond to treatment with early passage fresh MSC product while only 50% of the comparable patient populations show responsiveness to late passage frozen MSC treatment[29]. This is the first translational evidence, which demonstrate the clinical benefit of using fresh MSCs over heat-shocked thawed cells.

An important difference between academic and industry sponsored MSC clinical trials are the method of production, which may greatly affect fitness and function of transfused MSCs. The appeal of universal donor lot produced MSC is to manufacture large quantities of defined cells sufficient for multiple infusions. As an example, Prochymal®, used in the failed GVHD clinical trial from Osiris, was prepared from random donor volunteers with extensive (10,000 doses derived per volunteer donor) expansion. In contrast, academia-sponsored studies performed mostly in Europe typically use MSCs at earlier passages with manufacture of 5-10 doses per volunteer donor. Extensive cell culture on an industrial scale may be associated with aneuploidy and secondarily with replicative senescence, altered biology and reduced telomere length[30, 31]. In comparison to actively growing human MSCs, senescent cells fail to control lethal endotoxemia in animals and senescent MSCs do not inhibit lymphocyte proliferation efficiently and display migratory defects in response to proinflammatory signals[32]. It is unknown whether replicative senescent MSCs have intact IFNγ responsive machinery akin to early passage MSCs. Late passage MSCs are clinically less effective in ameliorating GVHD than the earlier passage MSCs[33]. However in an another clinical trial, difference in the clinical benefit was not observed between the treatment groups with MSCs from earlier and late passages[34]. As authors pointed out, the derivation of meaningful MSC effect in the setting of combination therapy is complex and more studies are required for the conclusion. Human fibroblasts, normal and tumorigenic epithelial cells become proinflammatory upon replicative senescence by acquiring a Senescence Associated Secretory Phenotype (SASP). The characteristic of SASP is the secretion of proinflammatory cytokines such as IL6, IL8 and GMCSF[35]. Thus further studies are required to determine whether senescent MSCs acquire a proinflammatory SASP-like phenotype and secretome. Culture expansion conditions can also influence the senescent behavior of MSCs since fetal calf serum can induce senescence relatively faster than the human platelet lysate culture conditions[36]. In addition, chemical analogues have been shown to increase the immunosuppressive properties of replication impaired MSCs[37]. It will be of future interest to characterize whether such molecules may rescue MSCs from high passage senescence.

2)Host Inflammatory Milieu and *in vivo* licensing of MSCs

Upon transfusion MSCs can be licensed by IFNγ derived from host lymphoid effector cells and deploy immunosuppressive properties[13]. Therapeutic efficacy of MSC was lost in a GVHD model when IFNγ KO donor T cells were used to induce the onset disease[38]. In a reciprocal experiment, it was shown that IFNγ receptor knock out MSCs do not exhibit any therapeutic activity against GVHD in mice, suggests that despite the presence of other synergistic cytokines, IFNγ licensing *in situ* is key for MSC mediated immune suppression[13]. In addition, *in vitro* studies demonstrate that the quantity of IFNγ determines MSC's proinflammatory and anti-inflammatory properties. At low IFNγ concentrations, MSCs become antigen-presenting cells while at the high concentrations they become suppressor cells[39]. Therefore, it is likely that the magnitude of available IFNγ but also the location of IFNγ availability, systemic or localized in the tissues and synergistic with the MSC's homing potential can influence the activation state of MSCs. IFNγ also upregulates MHC class II molecules by MSCs through the activation Class II transactivators (CIITA). Cell culture density and confluence tightly controls the regulation of CIITA molecules on MSCs. Stimulation with IFNγ on high-density MSC cultures upregulate increased levels of CIITA in comparison to low-density cultures[40]. This suggests that inter cell communication likely plays a role on MSC's effective responsiveness to IFNγ and suggests that cell dose and tissue homing might play an important role in their function. TNF-α and IL-1 can synergistically augment the effect of IFNγ on MSC's immunosuppressive properties[13] suggesting that MSC biology is responsive to environmental

cues and skews its immune plastic response accordingly. Conversely, we have shown that anti-inflammatory cytokine such as TGFβ blocks IFNγ responsiveness of MSCs[40], which provides insight on possible checkpoints to MSC immune modulation *in vivo*. It is also noteworthy that MSCs instruct the differentiation of $CD4^+$ T cells in to regulatory T cells through TGFβ and its role in MSC's therapeutic activity has been demonstrated in a mouse model of ragweed-induced asthma[41]. Although TGFβ skews IFNγ responsiveness, it will not abrogate MSC's suppressive characteristics. IL-2 is a proinflammatory cytokine which perpetuates T cell proliferation and prevents anergy. Exogenous addition of IL-2 reverses PDL1 mediated inhibition of T cells[29]. However addition of exogenous IL-2 does not abrogate MSC inhibitory potential *in vitro*. Whereas, blocking of IDO activity with 1-Methyl Tryptophan completely abolishes MSC's veto properties. Thus IDO expression by MSCs is necessary and microenvironmental IL-2 does not interfere with its suppressive effect on T-cells. Another host factor, which may influence MSC biology, is the concurrent use of immunosuppressive drugs to suppress immune responses in autoimmune disorders. MSCs have been shown to be act synergistically with mycophenolate mofetil and rapamycin to induce tolerance in solid organ transplantation models[42, 43]. Other common drugs used to treat inflammatory disorders such as azathioprine, methotrexate, 6-mercaptopurine and anti-tumor necrosis factor (TNF)-α, do not antagonize MSC's immunosuppressive properties[44]. In all these cases, immunosuppressive drugs act synergistically with MSC's immunosuppressive properties and no antagonism has been reported.

Priming of MSCs with IFNγ for clinical use

The compelling role of IFNγ on the veto function of MSCs supports the idea of using IFNγ licensed MSCs as a second-generation MSC therapy. Our studies demonstrate that IFNγ licensed MSCs but not resting MSCs have an inhibitory effect on T cell cytokine secretion in short term stimulatory assays[14]. Although resting MSCs inhibit T cell cytokine secretion in long-term cultures, this is dependent upon activation by T-cell produced IFNγ. Requirement of duration for this *in situ* licensing process can be bypassed through *in vitro* pre-licensing with IFNγ. For example resting MSCs have a therapeutic effect on GVHD only when they were administered on Day 9. In contrast IFNγ licensed MSCs have beneficial effect even if they are administered on Day 0[45]. In addition, it has been demonstrated that the degree of GVHD mitigation is directly associated with the magnitude of MSC's pre exposure to IFNγ[38]. Another study demonstrated that IFNγ pretreatment enhances MSC's capacity to inhibit Th1 inflammatory responses, resulting in diminished mucosal damage in experimental colitis[46]. These studies strongly suggest the benefit of utilizing MSCs pre-licensed with IFNγ. However, we have demonstrated that IFNγ licensed allogeneic MSCs lose their therapeutic activity against autoimmune encephalomyelitis (EAE)[47]. Considering that MSCs are not intrinsically immunoprivileged, random donor IFNγ licensed MSC are likely immune rejected by a host response to the upregulated expression of mismatched MHC class I and Class II molecules[48]. Thus, we speculate that IFNγ licensed allogeneic MSCs cannot serve as a "universal donor" in immunocompetent MHC-mismatched recipients and that cytokine pre-licensing would be of productive use only with HLA-matched or autologous MSCs. The therapeutic potential of transfused MSCs may not only rely on their anti-inflammatory properties but also on their homing and engraftment potential to inflamed tissue and sites of immune interaction. It has been demonstrated that IFNγ or IFNγ + TNFα licensed MSCs exhibit greater migration potential than resting MSCs to inflamed gut or ear in animal models[46, 49]. IFNγ licensing upregulates adhesion molecules such as ICAM-1 and VCAM-1 on MSCs, which can enhance the engraftment potential of MSCs. Interestingly the immunosuppressive role of these IFNγ inducible adhesion molecules is also demonstrated[50, 51]. Thus, engraftment and immunosuppression are inseparable properties of MSCs and are interconnected by IFNγ licensing.

Conclusions

Although there are unanswered question with regards MSC's origin and nature, their immunomodulatory abilities continue to be appreciated as an attractive cellular pharmaceutical. MSC's immunosuppressive and engraftment properties are highly dependent on their responsiveness to cytokine cues such as IFNγ. The methods used for cell manufacture and clinical delivery have substantial impact on the pharmaceutical fitness of MSCs for treatment of immune disorders. Considerations to employ early passage, metabolically fit, cytokine augmented MSC therapies will be of future clinical interest.

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Conflict of interests

None

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