Immunological characterization of multipotent mesenchymal stromal cells–The International Society for Cellular Therapy (ISCT) working proposal.

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Immunological characterization of Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy (ISCT) working proposal.

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Introduction

The large number of experimental approaches, culture conditions, qualitative and quantitative methods, and *in vitro* and *in vivo* models employed so far to assess immune regulatory properties of multipotent mesenchymal stromal cells (MSCs) has led to an excess of literature data that sometimes are poorly comparable, redundant, and even contradictory. Thus, quite paradoxically, the risk is that pre-clinical literature data may become eventually weak and scarcely useful, in both researchers' and Regulatory Authorities' opinion, for supporting experimentally specific MSC-based clinical trials aimed at treating autoimmune and inflammatory diseases. However, some data in this field appear more solid and reproducible and may be generally accepted to suggest reproducible immunological assays to quantify the differences in immune modulatory properties of MSCs produced according to Good Manufacturing Practice (GMP).

The MSC Committee of the International Society of Cell Therapy (ISCT) released a statement paper in 2005 that established the minimal criteria characterizing human MSCs (1), without focusing particularly on their immunological properties. In the 7 years following the publication of this statement paper, more than 10,000 manuscripts containing the term "mesenchymal stem cell" or "multi-potent mesenchymal stromal cell" in the title or abstract have been catalogued in PubMed, and many of them deal with immune regulation. To consolidate the scientific research in this field, the MSC Committee of the ISCT is publishing a working proposal paper aimed at stimulating the general discussion about the need of shared guidelines for the immunological characterization of MSCs for clinical use (**Box 1**).

1. MSCs as immune modulators and the assessment of regulatory properties

MSCs can be obtained from tissues that originate from distinct development programs and that contain distinct pools of endogenous progenitor cells. Therefore, the properties of tissue-specific MSCs should be carefully evaluated prior to their clinical employment. MSCs or MSC-like cells have been identified in bone marrow (2,3), adipose tissue (4), and many other tissues and organs (5) including lymphoid tissues (6,7). MSCs have been identified *in vivo* as peri-vascular cells expressing the STRO1, CD146 and 3G5 antigens (8-11). Despite a close relationship between these two cell types in terms of surface phenotype and qualitative *in vitro* assays (11,12), MSCs in general lack the contractility of pericytes and may show marked differences in gene expression (12), as well as by using more rigorous *in vivo* assays (13). In addition, some Authors have described a neuro-ectodermal origin of MSCs through either $Sox1^+$ neuro-epithelial cells (14) or $Nestin^+$ precursors (15). CFU-F–forming cells from bone marrow can be obtained through the prospective isolation of CD45-negative MSCs with anti-STRO1 (8), -CD271 (16), or -CD146 (17) antibodies, or selection for nestin-expressing cells (15). Nevertheless, not enough data concerning purified MSCs are available to assume that MSC progenitors/pericytes possess the same immune regulatory properties of *ex vivo* expanded adherent MSCs. Therefore, almost the entire amount of data concerning the immunological properties of MSCs refers to as adherent expanded MSCs.

It is now clear that MSCs of different tissue sources (6,7,18), as well as their stromal progeny (19), can interact with, influence and even profoundly affect the *in vitro* functions of most effector cells involved in innate or adaptive immunity (20). These properties have been the subject of many excellent reviews (21,22). However, some differences have been described amongst MSCs of different tissue origin (7,18); therefore, it cannot be presumed that all tissue-derived MSCs display equivalent immunoregulatory properties. We have a better appreciation that the *in vitro* and *in vivo* molecular mechanisms evoked can be influenced by several conceptual and experimental factors, including species and tissue sources of MSCs, culture conditions, number of passages determining culture-related senescence, activation status of both MSCs and responsive immune effector cells, analytical methods and animal models used (21-25). The obvious consequence of this heterogeneity in the scientific approach to MSC physiology is that very often *in vitro* and *in vivo* data are variable, if not contradictory and reciprocally not comparable. This conundrum is far from negligible: the assessment of the immune regulatory properties of MSCs is not merely a matter of biological speculation but has become the basis for the clinical use of these cells as cellular

immunotherapy in different conditions characterized by dysregulated allogeneic or autologous immune responses or simply abnormal defensive inflammation (26-32). National and international regulatory agencies typically require formal demonstration of the safety and effectiveness of the MSC-based treatment strategies in pre-clinical models. There is also an expectation that robust potency assays be developed and implemented as part of cell manufacturing, which may subsequently be associated with clinical effect and serve as a "gold standard" for inter-study analysis.

After clarifying the nomenclature for MSCs to avoid misunderstanding in comparing stromal cells (33), the field has sufficiently matured for a general discussion to reach shared guidelines for *in vitro* and *in vivo* methodologies defining the functional immune plasticity of MSCs. We have broad consensus that flow-cytometry phenotype analysis of MSC cellular products represents a minimum definition of identity. In the specific setting of MSCs used for immune modulation, the field has defined functionally relevant markers that merit attention and highlighted the importance of *in vitro* MSC licensing to further deploy a functional immune phenotype.

1.1. Resting versus primed MSCs: the role of MSC activation

In a resting state, MSC are at a default niche, displaying mostly bystander anti-apoptotic and immune homeostatic features biased toward suppression. These properties can be greatly enhanced when MSCs undergo functional polarization toward the inhibitory phenotype on exposure to various pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1α or IL-1β (24,25,34-43). A unique paradox of MSCs is their equal ability to behave as antigen-presenting and cross-presenting cells under similar conditions. Regardless, IFN-γ remains the first key licensing agent for MSC suppressor function. There is strong consensus that across species IFN-γ augments MSC suppressor function (through distinct effector pathways). *In vitro* MSC inflammatory "licensing" better recapitulates what likely happens *in vivo* once MSCs are transfused into patients with dysregulated immune responses or with systemic inflammation, including sepsis (25-32). In this context, Toll-like receptor (TLR) activation could strongly and variably modify MSC immune properties depending on the ligand, kinetics and strength of the stimulation (25). In fact, all measurable immunological features of MSCs, both at phenotypic and functional levels, depend on their activation status at the time of interaction with effector cells, although variability may be observed among different donors (36) and between fresh and thawed MSCs (38). Thus, if these functional assays aim at assessing the immune regulatory functions of MSCs for clinical purposes, comparing the results with both resting and licensed MSCs would be most informative, regardless of the species and tissue origin. Different protocols of MSC licensing are available

from the literature. IFN-γ is sufficient for licensing and should be used to deploy a functional phenotype; however, its effect is amplified by TNF-α. For instance, the addition of 100 IU/mL (10 ng/mL) IFN-γ and 15 ng/mL TNF-α for 40 hours of culture is adequate to obtain MSC activation before their challenge with immune effector cells (25,44); however, other licensing protocols may be suggested to trigger MSCs and make them become efficient immunomodulatory cells.

We suggest that standard immune plasticity assay be based on IFN-γ + TNF-α used as a model* in vitro *priming agent. The issue then arises about how MSCs are investigated after licensing.

1.2. MSC immunophenotyping as part of the immune plasticity response

Flow cytometry may be used to investigate the expression of cell-surface markers for characterizing MSC immunological properties. A traditional definition of a quiescent MSC is that of an MHCI-expressing cell lacking MHCII or co-stimulatory molecule expression (45,46). However, IFN-γ–primed MSCs robustly upregulate markers such as MHCI and MHCII molecules, immune modulatory molecules (CD200, CD274/PD-L1/B7-H1), cytokine/chemokine receptors (CXCR3, CXCR4, CXCR5, CCR7, CD119/IFN-γ receptor), adhesion molecules (CD54, CD106), DNAM ligands (CD112, CD155), NKG2D ligands (macrophage inflammatory complex [MIC] A/B, UL binding protein 1,2,3), and Notch receptors (Jagged-1) (21,22,24). Intriguingly, human MSCs do not upregulate co-stimulatory molecules (CD80, CD86) in response to IFN-γ, and immune modulators such as TGF-β can markedly blunt MHCII upregulation in response to inflammatory stimuli. MSCs express TLR-3 and TLR-4 and will respond to their respective agonists, double-stranded RNA (dsRNA) and lipopolysaccharide (LPS), which may also help in examining MSC immunomodulatory properties (24,47). MSC culture conditions and its impact on immune plasticity could be assessed as part of product characterization since the expression of many of these markers and implied immune plasticity may change as a result of culture conditions, post-cryopreservation and inflammatory priming (25,39).

As an aggregate suggestion, immunophenotypic analysis of an expanded cell product immediately before banking (if relevant), as well as at the time of transfusion to human subjects, may provide mechanistic insights into intra- and interstudy variation in clinical response among patients.

1.3. Culture modalities and *in vitro* cell responder assays

During *in vitro* MSC expansion, cell culture variables should be documented because of possible impact on immune plasticity response. Variables of interest are cell density at the time of passage, number of population doublings (in complementarity to number of

"passages"), culture medium (fetal bovine serum, platelet lysates, "defined" medium) and growth factors used. For co-culture experiments, especially with mixed lymphocyte reaction (MLR), MSCs can be used as either adherent cell monolayer or in suspension; in both cases different MSC/immune effector cell ratios should be tested to assess MSC veto functions on MLR. Both unselected peripheral blood mononuclear cells (PBMCs) or purified immune effector cells can be used, but the latter usually provide more reproducible results because of the lack of confounding third-party cells (monocytes in particular). A high viability of immune-responding cells is a crucial factor, given the anti-apoptotic activity of MSCs. Monocyte content of PBMCs from different normal donors may vary dramatically and this may lead to biased results due to different degrees of *in vitro* licensing process mediated by variable monocyte concentrations. Consequently, the use of purified immune cell subsets would minimize group variability in assays aimed at studying MSC immune regulation, unless a precise quantification of monocyte content, as a fraction of PBMCs, is provided. Concerning selection methods, immunomagnetic positive selection may lead to non-specific triggering of the target molecules, so indirect negative selection leading to highly purified samples (>95%) would be preferable (36). The technique used to activate responder cells may also have an impact on the measured potency of MSCs. Different types of stimuli have been used to trigger activation of immune effector cells in the presence of MSCs and range from MLR to specific antigens (21,41,42). For T cells, common stimuli are phytohaemagglutinin (PHA), a polyclonal stimulus leading to robust activation (20), and monoclonal antibodies against CD2, CD3 and CD28, which induce a more "physiologic" responder cell activation (43). This complexity, coupled with the use of unfractionated PBMCs versus purified T-cell responders, may add to the challenge in meta-analyzing intergroup results. In the human setting, a large number of MSC:immune effector cell ratios has been tested to assess the best coculture conditions to unravel immune regulatory effects, thus showing that the modulation of immune functions *in vitro* requires the presence of adequate numbers of MSCs. A ratio of MSCs to T cells of 1:5-10 is generally suitable to obtain a measurable effect (36,44), but a ratio of 1:1 or 1:5 would be preferable when MSCs are co-cultured with B cells (6,36,40,44) or natural killer cells (25,36,44,48,49).

For NK cells, allogeneic stimulation with irradiated target cell lines (i.e. K562) is commonly used, but is not always reproducible. Alternatively, IL-2 priming leads to strong NK cell activation and seems to give more reproducible results, although fresh or activated NK cells may have different efficiency in recognizing and lysing allogeneic MSCs (48,49).

MSCs can suppress immunoglobulin production by B cells (6,40,50). Cytofluorimetric evaluation of CD38/CD138 upregulation and parallel downregulation of CD20 seems to be a good approach to study the differentiation of memory B cells to plasma cells. ELISA or ELISpot can be used to monitor whole or specific immunoglobulin secretion by MSCs (51).

Different groups have studied a possible role of regulatory T cells (Tregs) in immunomodulation, with contrasting results (36,41,52). However, suitable assays to demonstrate the ability of MSCs to maintain or induce Treg expansion seem to be cytofluorimetric quantification of the proportion of CD4+/CD25+/FoxP3+/CD127- cells after co-culture with MSCs, and western blot and real-time PCR assay of FoxP3 expression, with functional evaluation of the regulatory potential of resulting T cells to fully confirm the results (52).

MSCs can also affect differentiation of monocytes to macrophages and dendritic cells, as well as their maturation, migration and functions. Monocyte differentiation can be studied by showing CD80, CD86 and HLA-DR upregulation and CD14 downregulation with activation (53). Maturation of monocyte-derived dendritic cells can be shown by studying the upregulation of MHCI and MHCII, CD11c and CD83 (53). Similarly, MSC effects on macrophages should be carefully characterized, as macrophages play a major role in many diseases, i.e. myocardial infarction, stroke and sepsis, for which MSCs have been suggested as possible therapeutic strategy. Assay of cytokine production (IL-10, TNF-α) and expression of macrophage 1 and 2 (M1/M2) surface markers, such as CD14, HLA-DR and CD206 may be used to evaluate the influence of MSCs on monocyte polarization towards macrophages (21,24,25,53-55). The activation protocol that seems to be more useful to discriminate between TLR-4-dependent MSC1 phenotype (MSC releasing mostly pro-inflammatory cytokines, such as IL-6, IL-8, or TGF-β) and TLR-3-dependent MSC2 phenotype (MSC producing immunosuppressive molecules, such as IL-4, IL-1RA, IDO and PGE2) is based on the short incubation (1 hour) with LPS (10 ng/mL) or poly(I:C) (1 µg/mL), respectively, followed by further 24–48 hour incubation in growth medium (24).

Only a few studies have investigated MSCs and neutrophil interactions (56,57). Human neutrophils, usually obtained from peripheral blood of normal volunteers, can be isolated with standard density-gradient separation methods (56) or high-purification procedures by positively removing all contaminating cells expressing CD3, CD56, CD19, CD36, CD49d, and Gly-A (57). The potential advantage of the latter method is that cell preparation is devoid of cells that might release factors influencing MSC and neutrophil functions regardless of their reciprocal interaction (57); consequently, high-purification procedure would be preferable to obtain more reproducible results. In any case, neutrophils should be manipulated under endotoxin-free conditions to avoid activation before co-culture with MSCs (56,57). Different stimuli (e.g., lipopolysaccharide, poly(I:C), phorbol esters), ratios of neutrophils to MSCs (from 1,000:1 to 10:1, in direct contact or in Transwell® conditions) and functional assays (e.g., CD16 and CD11b expression as surrogate markers of neutrophil viability and activation, respectively; ELISA for cytokine detection; superoxide anion release for respiratory burst quantification) may be used to assess the effects of MSC–

neutrophil interactions (56,57). The choice of one kind of stimulus rather than others would depend on which cell type needs to be activated to assess a specific effect. Poly(I:C) addition leads only to MSC activation via TLR3, as this receptor is not expressed by PMN (57), and consequently the observed phenomena do not depend on the simultaneous PMN activation. By contrast, LPS and phorbol esters activate both PMN and MSCs and the subsequent observed phenomena depend on the effects induced on both cell types. 100:1 and 10:1 PMN:MSC ratios and direct contact determine to the most evident effects on PMN survival, CD16 and CD11b expression, cytokine production and respiratory burst; all these phenomena may be assessed for a complete characterization of MSC effects on PMN; however, CD16 and CD11b expression could be used as surrogate markers of neutrophil viability and activation, respectively (56,57).

In summary, the use of purified responders (as opposed to unfractionated PBMCs) coupled with a more "physiologic" activation stimulus may be widely practicable and provide more generalizable guidance in examining the relative functional potency of MSCs and as a companion to clinical trials.

1.4. MSC cellular biochemistry

Activation of IDO and iNOS is a pivotal mechanism in lymphocyte inhibition with MSCs, but species-specific differences exist. For example, after inflammatory priming, human MSCs express extremely high levels of IDO and low levels of iNOS (23,58), which is opposite to that seen with mouse MSCs (59). The *in vitro* functional relevance of IDO bioactivity can be readily shown by use of the specific inhibitor L-1 methyltryptophan (L-1MT), which completely abolishes the inhibition of T-cell proliferation mediated by human MSCs (23,58). An array of potential complimentary suppressor pathways driven by MSCs includes heme oxygenase-1, soluble HLA-G5 and other secreted factors such as TGF-β, PGE2, galectin and tumor suppressor gene 6 (TSG-6) (21,25,53). Importantly, the MSC response to IFN-γ leads to increased expression of pro-inflammatory cytokines/chemokines such as IL-6, CCL2, CCL7 and CCL8. It is generally accepted that IDO bioactivity is central to the suppressor function of human MSCs and that IFN-γ regulation leads to massive transcriptional IDO induction.

Therefore, investigating the IDO response should be central to an in vitro regulation assay. Analyzing the transcriptional modulation of immune effector genes as part of the IFN-γ response could be further considered.

1.5. Animal models for *in vivo* assays assessing human MSC regulatory properties

Most *in vivo* experimental models assessing MSC immunomodulation are based on rodent species, with some exceptions involving non-human primates (60). Mouse models have been used to test the efficacy of MSC transplantation for the treatment of acute GvHD

(21,26,27,61-63), neurological (30,31) and systemic (32) autoimmune diseases, sepsis (28,55) and acute renal (29) and lung injury (64), as well as other pathological conditions (21). Two main issues should be considered when investigating the activity of MSCs in pre-clinical models: whether to measure their immunosuppressive potency or elucidate the mechanisms underlying the therapeutic activities.

Regardless of the general concerns about validity of mouse models, murine MSCs may have a significant number of differences as compared with human MSCs. Some differences involve culture conditions and modalities of their expansion, as mouse MSCs require a much longer time to expand under standard culture conditions than human MSCs. In terms of the molecular pathways of immunosuppression, human and mouse MSCs are relatively similar in the predominant use of the mammalian target of rapamycin pathway. However, human MSCs preferentially use IDO and murine MSCs selectively use iNOS in immunosuppression (23). IDO and iNOS are modulated by different inflammatory molecules; therefore, the murine microenvironment does not necessarily provide conclusive information about MSC regulation in patients. As well, caution should be used in interpreting data obtained from animal models related to the administration route. Intravenous injection of MSCs in mice is notoriously difficult because of the extremely high incidence of lethal pulmonary embolism, even with subtherapeutic doses. Some groups have used the intraperitoneal route, with variable outcome, because the trafficking of MSCs from this site has yet to be defined. The alternative approach has been to inject human MSCs into mice. Although human MSCs can improve a number of mouse models, most murine cytokines with MSC “licensing” activity do not cross-react with the corresponding human receptors, which may confuse the information able to be obtained.

The models may have fundamental biases, but the reproducibility of an animal model could be a great advantage in comparing different MSC preparations. In several conditions, including experimental arthritis and GvHD, the precise time for MSC administration for effective therapeutic activity has been defined. Therefore, an animal model would eliminate patient variability and facilitate an informative comparison of MSC lots and their potential manipulation. Although not applicable to a routine cell therapy laboratory, an initial *in vivo* characterization would set the stage to identify the main criteria for the selection of highly effective MSC preparations. Nevertheless, data obtained from *in vivo* studies in rodents should be critically evaluated.

Considering the substantial difficulties in obtaining clear and informative mechanistic and potency data from delivery of human MSCs to xenorecipient animal models, conclusions on how to conduct clinical trials should be drawn with caution.

Conclusions

Besides having regenerative properties for some tissues, cultured MSCs are mostly used in the clinical setting for regulating the immune response. The functional potency of MSCs should be quantified by standardized immune assays with purified responders as a scientific component of clinical trials. In the same way, immune cell populations could be prospectively analyzed in patients receiving MSC therapy. The final aim of methodological standardization is to obtain shared, reproducible and consistent data that may validate MSC-based clinical approaches as a potentially useful treatment for immunological diseases. The sincere hope of this working proposal paper is to contribute to the general discussion in the MSC scientific world, thus leading soon to shared guidelines and common protocols for the immunological characterization of MSCs for clinical use, in order to achieve comparable and unambiguous results on MSC efficacy in human diseases (65).

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Box 1

Suggestions for the assessment of regulatory properties of human MSCs

1. A standard immune plasticity assay should be implemented by using IFN-γ ± TNF-α as model *in vitro* priming agent.
2. Functional analysis of an expanded cell product may provide mechanistic insights on intra- and inter- study variance in clinical response amongst patients.
3. The use of purified responders would be widely practicable and should provide more generalizable guidance on relative functional potency of MSCs and as a companion to clinical trials.
4. Interrogating the IDO response as part of an *in vitro* licensing assay should be considered central.
5. Conclusions based on xenorecipient animal models on how to conduct clinical trials should be drawn with caution.
6. The prospective hypothesis-driven analysis of lymphocyte populations in patients groups treated with MSC should be encouraged.
7. Clinical analysis should also include the monitoring of whether injected MSCs are the target of an immune response.