Cytotherapy, 2017; 19: 784–797

International Society for Cellular Therapy
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REVIEW ARTICLE

Regulatory perspective on *in vitro* potency assays for human mesenchymal stromal cells used in immunotherapy

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Abstract

Mesenchymal stromal cells (MSCs) are multipotent cells derived from various tissues that can differentiate into several cell types. MSCs are able to modulate the response of immune cells of the innate and adaptive immune system. Because of these multimodal properties, the potential use of MSCs for immunotherapies is currently explored in various clinical indications. Due to the diversity of potential MSC medicinal products at the level of cell source, manufacturing process and indication, distinct functionality tests may be needed to ensure the quality for each of the different products. In this review, we focus on *in vitro* potency assays proposed for characterization and release of different MSC medicinal products. We discuss the most used functional assays, as presented in scientific advices and literature, highlighting specific advantages and limitations of the various assays. Currently, the most proposed and accepted potency assay for release is based on *in vitro* inhibition of T cell proliferation or other functionalities. However, for some products, assays based on other MSC or responder cell properties may be more appropriate. In all cases, the biological relevance of the proposed assay for the intended clinical activity should be substantiated with appropriate product-specific (non-)clinical data. In case practical considerations prevent the use of the ideal potency assay at release, use of a surrogate marker or test could be considered if correlation with functionality has been demonstrated. Nevertheless, as the field of MSC immunology is evolving, improvements can be expected in relevant assays and consequently in guidance related to potency testing.

Key Words: *cell-based therapy, government regulation, immunomodulation,* in vitro *potency assays, mesenchymal stromal cells, quality control*

Introduction

Mesenchymal stromal cells (MSCs) are multipotent cells that can differentiate into several cell types. MSCs can be isolated from various tissues and although their numbers are low and decrease with age, their potential to expand *ex vivo* may allow production of sufficient amounts for therapeutic use [1]. Human tissues from which MSCs have been isolated include bone marrow, adipose tissue, placental tissue, umbilical cords and embryonic stem cells [1–12]. Importantly, these MSCs differ in their expression of surface markers and in their functional capacity after stimulation with proinflammatory mediators [2,13]. As a consequence, comparison of the clinical effects of the various MSC products is hampered. To create a broader consensus for more uniform characterization of MSCs, the International Society for Cellular Therapy (ISCT) proposed three minimal criteria to define human MSCs: (i) MSCs should be plastic-adherent when maintained in standard culture conditions; (ii) MSCs should express CD105, CD73 and CD90 and should lack CD45, CD34, CD14/CD11b, CD79α/CD19 and human leukocyte antigen (HLA)-DR expression; and (iii) MSCs should be able to differentiate (*in vitro*) into osteoblasts, adipocytes and chondroblasts [14].

However, the nonspecificity of these criteria has been a topic of discussion ever since [15,16]. One should at least bear in mind that, in spite of above minimal criteria, MSCs remain heterologous populations of cells with a variety of gene expression profiles, differentiation and expansion potential and phenotype, which are influenced by tissue origin, cell isolation and expansion procedures [14,17–19].

In vivo, MSCs play a crucial role in peripheral tissue homeostasis (including blood vessels) and maintenance

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(Received 16 December 2016; accepted 21 March 2017)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2017.03.076

of the (hematopoietic) stem cell niche, mainly due to their potential to differentiate into various cell types and their secretion of several growth-promoting factors [20,21]. It has been shown that MSCs preferentially home to damaged tissue, where they are thought to exert their biological action by direct cell-cell interactions through their surface receptors, but also by producing soluble factors [18,22–24].

Apart from their tissue homeostasis and regeneration capacities, MSCs also have immunomodulatory abilities with potential therapeutic applications. This review article focuses on the *in vitro* testing of the functionality of these MSC medicinal products.

Immunomodulation by MSCs

The general immunomodulatory role of MSCs involves the orchestration of immunologic tolerance, next to the role of regulatory T and B cells and innate suppressor cells [25]. MSCs function via direct suppression of the activation, proliferation and effector functions of proinflammatory cells and the stimulation of various anti-inflammatory cell types to indirectly augment immune response regulation [20]. The most prominent MSC functions are exerted locally, although also some systemic effects have been found, e.g., via the induction of a more tolerogenic immune profile with an anti-inflammatory and T helper (Th)2-biased response [26–31].

To be able to exert immunosuppression, MSCs need to be preliminarily activated by proinflammatory cytokines like interferon gamma (IFN-γ) [32]. Inflammatory monocytes, which are initiators of inflammation, produce a proinflammatory milieu. Within this inflammatory environment, MSCs acquire an immunosuppressive phenotype and augment expression of their receptors and adhesion molecules (e.g., several integrins, pattern recognition receptors and cytokine receptors) to enhance interaction with other immune cells or even pathogens, contributing to a more efficient immunosuppression [1,6,19,21]. The suppression of cellular immunity by MSCs appears to be mainly based on paracrine effects via soluble mediators such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E_2 (PGE_2), transforming growth factor beta (TGF-β), nitric oxide, HLA-G5 and several interleukins (ILs), which are released after cross-talk with activated immune cells [19,33–35]. MSC-mediated modulation of the (local) immune response is the result of cumulative action of several of these soluble mediators, because none of these factors alone can completely abrogate lymphocyte proliferation for example [32].

MSC-mediated modulatory action on specific cells of the immune system has been extensively investigated and described [6,13,18,19,21,26,35–41]. They can exert this effect both on the innate (a.o. natural killer cells, neutrophils, monocytes, macrophages and dendritic cells [DCs]) and on the adaptive (T and B cells) immune system. The effects of MSCs on innate cells includes inhibition of their maturation, changes in cytokine secretion profiles and differentiation toward a more tolerant or regulatory phenotype.

The consequence of MSC actions on the function of T cells is somewhat controversial and conflicting results have been reported, possibly due to differences in the MSC culture method and tissue of origin. Effects of MSCs on $CD4^+$ T cells mainly involve inhibition of proliferation via cell cycle arrest in the G_0/G_1 phase, alterations in Th subtype proportions and induction of regulatory T cells (Tregs) to tip the balance toward a more anti-inflammatory response [19,37,39]. Importantly, most studies only determined the effect on cytokines produced by T cells as parameter for MSC functionality, whereas other T cell properties (e.g., chemotactic potential) have only incidentally been analysed [42]. For $CD8^+$ T cells, the effect on functionality (among which is cytotoxicity) is more clear; MSCs can only suppress the stimulation of antigen-specific cytotoxic T cells, but they do not inhibit previously activated (memory) cells and inhibition of the effector functions of $CD8^+$ T cells is only possible when they are not yet in their cytotoxic phase [18,39,43].

Conflicting results have also been described for the modulation of B cell responses by MSCs. Some authors have shown inhibition of proliferation, impairment of antibody secretion and changes in chemotactic properties, whereas others have found opposite results [18,19,21,32]. As with data obtained with T cells, these contradictory results are most likely the consequence of differences in the specific MSCs and/or in experimental conditions used.

Overall, MSCs skew the inflammatory environment into an anti-inflammatory one, both directly and indirectly, through immunoregulatory circuits involving a.o. monocytes and Tregs [35,36]. The potential of this immunomodulatory capacity is considerable, as MSC-mediated immune modulation can even cross species barriers, although the main mechanisms of action may differ between species [18,44].

MSC product development

Clinical application

As said, MSCs have a diversity of physiological functions including the ability to migrate to inflamed tissue, differentiate into various cell types and secrete anti-inflammatory and tissue-renewing factors. Due to their broad immunoregulatory potential, clinical application of these cells is explored for a wide range of disorders with high immune activation, including graft-versus-host disease (GvHD), transplant rejection and

autoimmunity [26]. Because MSCs harbor low immunogenicity (despite displaying HLA class I expression), potentially due to their immunosuppressive capacity, allogeneic cells may be used in acute conditions as "off-the-shelf" products, meaning that a sufficient number of cells is immediately available when required [45]. However, for disease conditions where rapid intervention is not needed (e.g., autoimmune conditions), the use of autologous cells is still preferred, except when the functionality of these autologous MSCs is impaired by the disease [32].

MSC efficacy is widely evaluated in clinical trials; at least 700 MSC trials were registered on www.clinicaltrials.gov in 2016 [19,23,33]. Unfortunately, results between studies are difficult to compare, due to differences in the utilized MSC products. Much is still unknown about the clinical effects of MSCs, e.g., combination therapies with other immunomodulatory drugs or the duration of the response after single or multiple cell injections [39]. Moreover, the immunomodulatory function of MSCs may depend on the degree of inflammation present in any given *in vivo* environment and, therefore, MSC preparations are expected to exert various degrees of immunomodulation in different patient populations and clinical indications [13,46]. Finally, properties of individual MSC products may vary due to reasons such as differences in tissue origin and manufacturing process and could potentially influence immunomodulatory function. It is therefore essential to ensure that relevant quality attributes of MSC products are adequately controlled.

Quality control

The quality of MSC products is broadly ensured at three levels: the selection of the starting material, the control of the manufacturing process (including Good Manufacturing Practice (GMP) and in-process testing) and the final release testing of the product. Quality control testing during manufacture and before release includes a.o. identity, purity, safety and potency. Potency is the most challenging parameter to test for in cell-based therapeutic products because it should reflect one or more of the cells' relevant *in vivo* functions [47,48]. Thus, a potency assay should be an assay based on functionality and not solely based on cellular phenotype. In addition, because the assay should be able to differentiate between sufficiently potent and subpotent batches, a (semi-)quantitative assay is required.

Both the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA) have provided general guidance for potency testing of cell-based therapies, but no cell type–specific tests have yet been proposed [48–51]. In this review, we provide an overview of *in vitro* potency assays that have been suggested for characterization and release for MSC medicinal products as a measure of their immunomodulatory function. For the functional assays most widely used and presented in the literature, advantages and limitations are discussed (summarized in Table I). In addition, we discuss potency assays that have been the subject of scientific advice for allogeneic MSC medicinal products in development.

In vitro assays to test for MSC functionality

As said, three minimal criteria for defining human MSCs have been proposed [14]. In addition, Wuchter *et al.* posed several criteria for GMP-grade MSCs [74]. However, although the criteria address MSC functionality, no concluding recommendations regarding potency assays are provided. This is in line with the notion that every MSC product is unique and that quality control assays should be justified on a case-by-case basis. Nevertheless, the following overview of potency assays to test for MSC activation status and MSC-mediated inhibition or induction of specific responder cells can provide valuable clues for the selection of appropriate potency assays for MSC products.

Assays to test MSC activation status

Cell viability and phenotypic characterization by e.g. surface marker expression are often regarded as (part of) both identity and potency testing for MSC products. Although testing of these parameters is required to ensure the production of a pure, viable population of the desired cell type, their relationship with *in vivo* functionality of a MSC product has not been unambiguously demonstrated. Nevertheless, for several actual MSC medicinal products, phenotypic characterization of the cells is the only potency assay presented.

Several cellular markers have been proposed as indicator or even as surrogate marker for MSC immunosuppressive capacities. One example is the expression of CD200 on MSCs. Binding of this glycoprotein to its receptor on other (mainly activated) immune cells has been shown to lead to the induction of inhibitory signalling pathways in these cells [52]. CD200 is constitutively expressed on MSCs, although the expression varies between MSCs derived from different sources and is modulated by proinflammatory cytokines, which would imply that the extent of CD200 expression may be a (semi-)quantitative measure of functionality. However, Najar *et al.* found that blocking CD200-CD200R interactions did not impair the suppressive capacity of the studied MSC product [52]. Thus, to what extend CD200 expression can be considered a surrogate

Table I. Overview of the advantages and limitations of frequently proposed *in vitro* potency assays.

Proposed *in vitro* potency assay	Stimulator cells	Responder cells	Additional stimuli	Read-out parameter	Advantages	Limitations	Key references
MSC activation assay	—	MSCs	Proinflammatory cytokines (e.g., IFN-γ, TNF-α or IL-1β)	Expression of intracellular and surface proteins (CD markers like CD200, cytokine receptors like TNF-αR2, IFN-γR1) Soluble mediator production (e.g., TGF-β, TNF-αR1, kynurenine, PGE_2)	Assays measure important surface or secreted factors involved in MSC functionality. Assays are relatively straightforward to perform.	Surrogate markers of functionality (although usually correlates with inhibition of T cell proliferation). Only focussing on one immunomodulatory mechanism.	[10,52–55]
Immune cell inhibition assay (short)	MSCs (or their lysate or conditioned medium) or other immune-modulatory cells	PBMCs T cells	LPS (after incubation with MSC lysate/medium) PHA aCD3/aCD28	(Stimulation or inhibition of) cytokine production (e.g., IL-10 and IFN-γ) (Inhibition of) T cell surface marker expression (e.g., CD25, CD154 or CD69)	Short assays (2 days). Assays measure the functionality of MSCs on an important cell type (T cells).	Surrogate markers for suppression of T cell functionality (although usually correlation with long-term assays). Assays do not directly address the impact on the effector function of T cells (except for read-out of cytokine production).	[53,54, 56–59]
Immune cell inhibition assay (long)[a,b]	MSCs	PBMCs T cells	Memory antigen (e.g., tetanus toxoid, HA or CMV) PHA PMA/ionomycin aCD3/aCD28(/IL-2) ConA	(Inhibition of) cytokine production (e.g., TNF-α and IFN-γ) (Inhibition of) T cell surface marker expression (e.g., cytokine or chemokine receptor) (Inhibition of) T cell proliferation	Assays measure the functionality of MSCs on an important cell type (proliferative or effector function of T cells). Assay culture time is usually shorter than MLR (3–4 days).	Assays can lead to misleading results (due to cell death or functional alteration of cultured cells). Assays do not directly address the impact on the effector function of T cells (except for read-out of cytokine production).	[4,5,7,12, 27,31,43, 55,60–72]
Immune cell inhibition assay (MLR)[a]	MSCs	PBMCs T cells	Allogeneic PBMCs Allogeneic DCs	(Inhibition of) cytokine production (e.g., TNF-α and IFN-γ) (Inhibition of) T cell surface marker expression (e.g., cytokine or chemokine receptor) (Inhibition of) T cell proliferation	Assays measure the functionality of MSCs on an important cell type (proliferative or effector function of T cells). Assays more natural mimic of antigen presentation (compared with mitogen- or antibodies-based assays).	Long assays (6–7 days). Assays can lead to misleading results (due to cell death or functional alteration of cultured cells). Assays do not directly address the impact on the effector function of T cells (except for read-out of cytokine production). Assays do not address the effect on third-party cells.	[4,5,8,12,63, 65–67,69, 70,72,73]
Immune cell inhibition assay (xenogeneic)	MSCs	Murine antigen-specific T-cell clones	Cognate antigen (and APCs)	(Stimulation or inhibition of) cytokine production (e.g., IL-10 and IFN-γ) (Inhibition of) T cell surface marker expression (e.g., CD markers) (Inhibition of) T cell proliferation	Low assay variability. Assay has the ability to compare the function of different human MSC donors or effects of variations in culture.	Assay does not make use of species-specific responder cells. Antigen used is not disease-related.	[44]

(*continued*)

Table I. Continued

Proposed *in vitro* potency assay	Stimulator cells	Responder cells	Additional stimuli	Read-out parameter	Advantages	Limitations	Key references
Immune cell migration assay	—	T cells (after MSC contact)	Chemokines (e.g., CXCL10)	(Inhibition of) T cell chemotaxis	Assay measures the functionality of MSCs on an important cell type (effector function of T cells).	Assay measures only one T cell effector function (which may be less relevant at the site of inflammation).	[42]
Suppressor cell induction assay	MSCs	Immune cells (e.g., T cells)	PHA aCD3/aCD28 and cytokines (mainly IL-2, TGF-β)	Treg induction (cell surface marker expression, cytokine production and suppressive function)	Assay measures the functionality of MSCs on an important cell type (T cells) to augment suppressive function.	Assay measures only induction or functionality of one T cell subtype.	[4,63]

TNF, tumor necrosis factor; LPS, lipopolysaccharide; PHA, phytohemagglutinin; aCD3/aCD28, anti-CD3/anti-CD28; HA, hemagglutinin; CMV, cytomegalovirus; PMA, phorbol myristate acetate; ConA, concanavalin A; APCs, antigen-presenting cells; CXCL, C-X-C motif chemokine ligand.

[a]References are literature examples where these very similar assays are described.

[b]Some studies used allogeneic responder cells, but induced T cell activation/proliferation with a-specific stimuli such as PHA.

marker for the immunomodulatory functionality of MSCs will need further investigation.

Another example of a proposed surrogate marker is tumor necrosis factor alpha receptor (TNF-αR) expression on MSCs [53]. A major trigger for MSC activation and migration is TNF-α and, as analysis of TNF-αR expression on MSCs or secretion of soluble receptors in supernatant is a relatively straightforward test to perform, it may be an attractive marker for the functionality of MSCs. If the level of TNF-αR expression correlates with the amount of inhibition of T cell proliferation, this could be a useful surrogate marker as release potency assay, provided that clinical relevance can be shown and receptor expression and the effects on responder cells are appropriately characterized.

In addition, IDO expression may be used as a surrogate marker for MSC functionality. IDO is one of the most important factors for human MSC-mediated immunomodulation [10,34]. This intracellular enzyme catalyses tryptophan degradation toward kynurenine and depletion of tryptophan abolishes T cell proliferation [75,76]. However, IDO is not expressed in resting MSCs and IDO activity is usually time-dependent in *in vitro* co-cultures of MSCs and responder cells. Aggarwal and Pittenger stated that IDO activity may only contribute to MSC-mediated T cell inhibition at later time points, making analyses of this activity in short assays (e.g., using mitogen-stimulated T cells) unreliable [26]. It is more likely that factors like PGE_2 exert the MSC suppressive function in these short assays. Nevertheless, short-term experiments by Menta *et al.* demonstrated that IDO expression and activity could be measured, indicating that culture and activation conditions of both MSCs and responder cells determine whether or not an immunomodulatory factor can be tested [10].

Although surrogate markers for immunomodulatory function of MSCs could be useful, potency evaluation in characterization studies based solely on such markers is not sufficient and should include an actual bioactivity assay. However, the use of a marker as a surrogate for MSC functionality may be considered as a release test, as long as appropriate justification is provided, supported by characterisation and/or (non-)clinical data. Because none of the effector molecules secreted or expressed by MSCs can induce the required immunomodulation all by themselves, clear evidence should be presented showing that (i) the surrogate marker is linked to an effect at cellular level (e.g., decreased T cell proliferation and adapted T cell polarization), (ii) it is correlated with relevant clinical effects and (iii) that via this expression the assay can detect clinically relevant defects and subpotent batches as these could occur in the specific manufacturing process.

Assays to test MSC-mediated inhibitions of immune cells

The most commonly proposed potency assays for characterization and release of MSC products involve the inhibition of T cell activation or proliferation in a co-culture with MSCs. Induction of T cells is usually achieved with memory antigens, mitogens (e.g., phytohemagglutinin [PHA], phorbol myristate acetate [PMA] or concanavalin A [ConA]), T cell receptor cross-linking and co-stimulation (anti-CD3 [aCD3]/anti-CD28 [aCD28]) or via the effect of allogeneity (e.g., allogeneic peripheral blood mononuclear cells [PBMCs] or DCs in a mixed lymphocyte reaction [MLR]).

Both mitogen- or aCD3/aCD28-based assays and MLRs have their specific advantages and limitations. An advantage of mitogen- or antibody-based assays is the relative short culture period (about 3–4 days). Nevertheless, the T cell activation is a-specific (with induction of most of the T cells), while the use of memory antigens would induce an antigen-specific response that more resembles the *in vivo* situation. Also, a MLR is considered to mimic an *in vivo* response like the one found with e.g. GvHD, which is one of the most important indications for MSC therapy. And in the recent past, the MLR was considered to be the golden standard for MSC potency testing. However, the assay also has some drawbacks. First, it takes 6–7 days before read-out can occur. Second, the induction of T cell proliferation is largely dependent on the histocompatibility (mismatch) between the stimulator and responder cells, making a relatively high assay variability inevitable. In addition, it appears that, possibly depending on their tissue-origin, MSCs may have some form of immunogenicity, indicating that at least part of the MLR results could be the consequence of a mismatch between MSCs and responder cells [9,77]. This implicates that for products containing allogeneic MSCs (which is the majority), it should be shown that the results of the potency assay are not due to display of alloreactivity, or the use of an alternative T cell inhibition assay could be considered.

For all types of assays the results are partially determined by the age, gender and infection history of the MSC donor. These differences increase variability in the assay outcome (and could also mask minor differences between batches), whereas they may not impact clinical efficacy [44].

Responder cells

The responder cells used in immune cell inhibition assays are mostly isolated total $CD3^+$ T cells or subsets like $CD4^+$ T cells. However, discussion about the use of specific cell types versus total lymphocyte or mononuclear cell populations is ongoing and the ISCT stated that the use of T cells as responder cells will overlook the impact of MSCs on other cell types [78]. Nevertheless, they also acknowledged an important disadvantage of using a mixed population of cells instead of a more pure population: although PBMCs are more representative of the *in vivo* environment where MSCs need to exert their function, the interaction between the different cell types would make the potency assay more complex and would inevitably lead to more variability [78]. Thus, using purified T cells (or even a T cell subtype) rather than PBMCs could be a better choice in a MSC potency release test. But even then assay variability caused by heterogeneity of the responder cell population should be acknowledged. For instance, it has been shown that the frequency of specific $CD4^+$ subpopulations (e.g., Th1 and Th17) within a T cell responder batch correlates with suppression [25]. The amount of these subpopulations, but also the amount of Tregs, may significantly differ between donors. Tregs have an intrinsic suppressive capacity and are further stimulated by MSCs and differences in the relative amount of Tregs in a T cell responder batch could lead to underestimation or overestimation of MSC immunomodulatory potential. Therefore, to improve assay variability, acceptance criteria for the percentage of different subpopulations within the responder cell preparation may be needed. Alternatively, if feasible, the same responder cell preparation could be used throughout the products' lifecycles. Obviously, assay variability due to the choice and control of the responder cells should be part of the robustness validation of the assay. The choice of responder cells should be justified based on product characterization studies, for example, with respect to the interactions between different immune cells and MSCs.

Read-out parameters

The common read-out of immune cell inhibition assays for MSCs is the suppression of T cell proliferation. The use of inhibition of activation marker (e.g., CD25, CD69 or CD154) expression as read-out, which would only require culturing for a few hours and which is used in other suppression assays, may not be appropriate for MSCs [53,56–58]. The main concern is that the antiproliferative effect of MSCs appears not to be due to inhibition of T cell activation. Studies in mice by Glennie *et al.* suggest that MSCs induce a cell cycle arrest in activated T cells without affecting the expression of early activation markers CD25 and CD69 [41]. This phenomenon was also found with several studies using human MSCs [43,60]. On the other hand, the effect on T cell activation markers could be dependent on the MSC culture conditions and the presence of other cells in the co-culture with T cells [61,79]. Therefore, depending on the MSC product, inhibition of activation markers as read-out of an immune cell inhibition assay could be justified.

Next to activation and proliferation, other parameters related to functioning of responder cells (e.g., cytokine production and chemotaxis) could be taken into account [42,54,59]. Nevertheless, analysis of e.g. a specific cytokine produced by T cells (more IL-10, less IFN-γ) is only considered a surrogate marker for MSC functionality; effects on T cell cytokine response alone are very difficult for predicting whether MSCs will reduce or augment an inflammatory response because the amount of cytokines (related to Th1, Th2 and Th17 cells), the timing of production and the duration of exposure influence the final outcome [20].

Finally, in read-out of the results of the immune cell inhibition assay, one should keep in mind that in some cases measured suppression is not necessarily the consequence of functional MSCs, but a result of a flaw in the assay. For example, competition for nutrients in culture medium and unexpected cell death could both lead to misleading amounts of suppression. Appropriate controls for cell concentration and viability are therefore essential. Addition of cell types other than MSCs may be considered as a control for MSC-mediated suppression.

Variations to standard inhibition assays

As outlined earlier in this article, due to the nature of the immune cell inhibition assay, it shows considerable variability, for example, as a result of culture conditions and responder cells. In the literature, several variations to the standard inhibition assay were described and aimed to improve consistency.

For example, Nazarov *et al.* proposed the use of antigen-specific T cells derived from mice in a standard inhibition assay [44]. This assay would probably not be feasible for release because the responder cells are quite artificial and acquisition of antigen-specific T cells from T cell receptor-transgenic mice is labor-intensive. However, the unlimited availability of these murine T cells reduces the variability of the assay and, therefore, such a test could easily compare the potency of different MSC batches or the effect of e.g. different culture conditions on the MSC function. Along the same line, Ketterl *et al.* recommended to pool cells from several donors as responder cells, which would intrinsically lead to a MLR response [5]. The pooling would lead to a sufficient amount of responder cells for many more potency assays than would be possible with cells from a single T cell donor, and could thus reduce variability due to difference in e.g. proliferation potential of T cells derived from different donors. However, pooling will not be ideal in all situations; as in case of an autologous MSC product, the responder cells should preferably be autologous as well, although this would introduce a substantial amount of variability and setting appropriate specifications for the potency assay would be more difficult. In addition, the acuteness of intervention when applying autologous MSC products may prevent the use of more complex assays with autologous responder cells.

Another way to improve assay consistency proposed by Bloom *et al.* consisted of the addition of a titration curve from suppression results of five different MSC to responder cell ratios to a standard inhibition assay (aCD3/aCD28-stimulated PBMC and MSC co-culture) for the calculation of a single "immunopotency assay value" [62]. In addition, they used a MSC reference standard to ensure the reliability and reproducibility of the assay and minimized assay-to-assay variability by using a single PBMC donor. As pointed out by the authors, this assay would be very useful to titrate the T cell activation stimulus or to investigate the effect of additional cell types or soluble factors. Making use of this specific immunopotency assay value would result in a more reliable outcome of the *in vitro* assay, although the feasibility of this assay for release is questionable when only a small amount of cells is available for testing, for instance, in case of autologous products. Moreover, the reference preparation should be carefully selected, especially in case a pool of MSCs from different donors is used, as proposed by Ketterl *et al.* [5]. Such a pool should consist of a randomly chosen combination of donors because preselecting on highly potent batches for the reference preparation may result in the rejection of less (but sufficiently) potent batches.

Assay standardization and robustness could be further improved by using a reference cell line. Salem *et al.* discussed the use of the widely available human T cell line Karpas 299 as a reference immunosuppressor in a MLR to define the relative suppressive capacity of specific MSC batches [25]. However, for a release assay it would be more useful to express results as percentage inhibition of activation. In addition, the Karpas 299 cell line more resembles Tregs than MSCs [80], and suppressor to responder ratios used in this assay are far from physiological. Therefore, the use of K299 as a reference is dubious and an in-house MSC standard would be preferred. In general, physiologically relevant ratios between MSCs and responder cells should be analysed in the potency assay when possible, otherwise the effect is likely not relevant *in vivo* [18,23,81].

Besides variability, improvement of the immune inhibition assay for MSCs could also be achieved by closer mimicking the potential *in vivo* action of MSCs. Kimbrel *et al.* suggested a two-way MLR by replacing the T cell activation stimuli (e.g., aCD3/aCD28) by allogeneic DCs [8]. This will introduce the analysis of an extra immunomodulatory function of the MSCs, a.o. the inhibition of DC maturation, but also an increase in the variability and complexity of the

assay. Only the standard use of a single donor for the responder T cells and a single donor for the DCs (thereby preventing differences in histocompatibility between assays) would make such an assay suitable as release test for MSC products. This would mean that both responder cell types should be stored in sufficient amounts to perform many potency assays. To preserve the consistency of this assay, freezing and thawing should have no significant effect on the HLA expression and differentiation status of the responder cells [6].

Assays to test MSC-mediated activation of suppressor cells

Apart from direct suppressive activity, MSCs can also indirectly modulate proliferation and functioning of effector cells. Promoting the survival of monocytes and inducing their differentiation toward type 2 macrophages is a key step in MSC-mediated generation of a more anti-inflammatory milieu [36]. These monocytes appear to be indispensable for the induction of Tregs [38]. In turn, Tregs enhance monocyte differentiation toward type 2 macrophages, which results in a further amplification loop for Treg formation and augmentation of the MSC effect on the on-going immune response.

Several studies have found that MSCs can induce suppressive Tregs from naïve effector T cells. There is evidence that MSCs can also directly activate naturally occurring Tregs *in vivo*, which may be of great importance when therapeutic MSCs would only be short-lived [82]. Sivanathan *et al.* co-cultured effector T cells with MSCs and revealed induction of Tregs from these effector T cells, probably due to the production of cytokines like TGF-β [63]. The induced Tregs were found to functionally suppress effector T cell activation. Bárcia *et al.* performed comparable co-culture assays to induce Tregs, although the percentage of Treg induction differed significantly between MSCs derived from different tissues, which was largely in line with a study from Mareschi *et al.* [4,27]. This implicates that the effect of MSCs on Tregs determined in a reliable and well-controlled assay could be considered as a measure of potency for MSC products.

Efforts to correlate in vitro *potency with* in vivo *immunomodulation*

Although various potency assays for the determination of MSC functionality have been proposed, as described in the previous sections, only a few studies have tried to link the *in vitro* assay to the *in vivo* immunomodulatory capacity of the respective medicinal product.

In studies by Bárcia *et al.* and Roemeling-van Rhijn *et al.* it could be demonstrated that differences in *in vitro* potency between different MSC types (i.e., umbilical cord tissue-derived, adipose tissue-derived and bone marrow-derived) correlated with *in vivo* potency in an animal model [4,55]. Because characterization results of these MSC types were largely comparable, the outcome of the assays can be considered as a first step in the assessment of batches with different potencies to link the *in vitro* read-out to *in vivo* MSC efficacy. Nevertheless, it is noted that in some of these studies inactivated cells were used in the *in vitro* assay but not in the *in vivo* study [4,9]. Yet, the effect of MSC inactivation (via radiation or chemically) on their immunomodulatory function should be further investigated to better evaluate the actual relation between the *in vitro* and *in vivo* activity.

In a more recent study from Bárcia *et al.*, the effect of cryopreservation and thawing on the viability, immunophenotype and immunomodulatory activity of umbilical cord-derived MSCs was studied [64]. The therapeutic potency was first tested *in vitro* with a T cell inhibition assay using three different MSC batches, which resulted in a dose-dependent suppression of the responder cell proliferation. Next, MSCs were used in an arthritis model to diminish both local and systemic inflammatory signs. Although it was not clear whether the same drug product batches were used for the *in vitro* and *in vivo* assessment of MSC functionality, this test combination may be very useful to correlate *in vitro* potency results with *in vivo* immunomodulatory activity. Nevertheless, because no significant impact of cryopreservation and thawing on the potency of the MSC product was found in this study, the determination of this correlation could be further improved by using both potent and subpotent batches in the *in vitro* assay and for *in vivo* treatment.

Most studies did not allow any conclusions on a possible quantitative correlation between *in vitro* and *in vivo* assay results, whereas others did not find a correlation between *in vitro* potency and *in vivo* immunosuppressive capacity [8,31,65–67,77]. In one of these studies it was shown that low IL-6-secreting MSCs had impaired *in vivo* functionality, while these cells showed sufficient suppression of proliferation *in vitro* when a high MSC to splenocyte ratio (but not a low ratio) was used [31]. This demonstrates the importance of characterization studies to select the most relevant *in vitro* potency assay or sets of assays. Subsequently, further insight in the correlation between the selected *in vitro* measure(s) of potency with the *in vivo* MSC functionality may be obtained by comparison of subpotent and potent MSC batches. Such data would be very informative, although it should be taken into account that effects found in animals do not necessarily reflect the human situation. Therefore, it also remains important to demonstrate that the batches that are considered sufficiently potent in the

selected *in vitro* potency assay(s) are the batches with clinical benefit in humans.

Selection and design of potency assays

Despite the common use of immune cell inhibition and suppressor cell activation assays, there is still a lot of uncertainty with respect to which assay would be most appropriate to assess potency of MSC products. For instance, using a potency assay combining both MLR and mitogen stimulation, Ketterl *et al.* observed that some MSC batches showed little suppression in the mitogen-stimulated assay, while showing a much higher suppression in the MLR [5]. This would indicate that a specific batch would have been released with one assay while being rejected with the other. This exemplifies the importance of comparing several assay approaches during development and choosing the most relevant and robust potency marker for release testing. In this respect, it is noted that the ISCT addressed the issue of identification of functional potency markers and declared that it is still unknown whether a MLR truly reflects the mechanism of action (MoA) of human MSCs *in vivo* [78,83]. In line with this and already mentioned earlier, a study from von Bahr *et al.*, where patient PBMCs were stimulated with a pool of five irradiated allogeneic PBMCs (=MLR) or with PHA and subsequently co-cultured with irradiated MSCs (10:1 ratio), showed that there was no correlation between the results of these *in vitro* assays and the clinical outcome [66]. This suggests that this standard T cell inhibition testing would not be a reliable potency assay for release, although this should be assessed on a case-by-case basis, as different manufacturing processes may produce MSCs with distinct functionalities *in vitro* and *in vivo*.

To be able to develop an appropriate potency assay and to justify the relevance of this assay for release, extensive characterization of the MSC product is necessary. In addition, data from (pre-)clinical studies will be required to show clinical relevance of the assay chosen to analyse MSC functionality. Because MSC medicinal products are all unique, justification of the potency assay can not be solely based on data generated with other MSCs, although results obtained with comparable products can form a starting point for further product-specific studies. Based on the results of product-specific (pre-)clinical studies and, if appropriate, prior knowledge or literature data, a picture of the clinical action of the MSC product should try to be obtained. This picture may be based on scientific assumptions instead of conclusive data because *in vitro* and *in vivo* models can not completely represent the human situation.

The immunomodulatory function of MSCs may involve many molecular mechanisms, as described earlier in this review. However, it is expected that the main mechanism by which MSCs in the product will exert their *in vivo* function is determined. This includes the identification of the key disease-related cell type that is affected by MSC activity and the main effector molecules that play a role in this immunomodulation. In addition, the issues mentioned below should be taken into account in the design of the potency assay used for release and/or characterization.

Choice of the assay type

The first decision in the development of an appropriate potency assay is the choice of the assay type, mainly whether to focus on immune cell inhibition or suppressor cell induction. The relevance and acceptability of the chosen potency read-out largely depends on the clinical indication and the anticipated therapeutic effect. For example, autoimmune disorders are a group of conditions for which a potency assay based on the induction of suppressive Tregs could be seen as an appropriate test, because failure of Tregs to control immune tolerance has been suggested as a principle mechanism behind autoimmunity [84]. In this setting, the anticipated therapeutic effect would be based on its potential to induce functional Tregs to restore homeostasis [36]. If so, then a potency assay based on stimulation of Tregs (suppressor cell induction) rather than direct suppression of autoreactive effector T cells (immune cell inhibition) may be most appropriate. More general, per MSC product, (pre-)clinical studies and scientific knowledge will have to predict which cell type is most affected by the MSCs and would be the responder cell of choice in the potency assay. Moreover, when MSC batches from the same manufacturing process would be used for two different indications with different mechanisms of action (e.g., GvHD and wound healing), potentially two different potency tests for release could be required.

Choice of the responder cell type

The responder cell type in the potency assay should be the key disease-related cell type affected by MSC activity. As said, potency assays usually involve $CD3^+$ T cells as responder cell type. Nonetheless, it is not yet known whether individual T cell subtypes are susceptible to different MSC mechanisms or whether the suppressive mechanism varies by culture conditions and/or disease pathogenesis [39]. Therefore, in characterization studies also the effect on specific subtypes (e.g., $CD4^+$ or $CD8^+$ T cells, naïve or memory cells) may have to be determined to get a more reliable insight into the product-specific mechanism of action [7,12]. Nevertheless, it may not be necessary to use a specific T cell subset (instead of the whole T cell population) in the potency assay because analysis of the MSC effects on the different subsets can be determined afterward

when using e.g. flow cytometry. However, this requires the selection of appropriate phenotypic markers for the read-out of the assay because different cell types may have overlapping phenotypes (e.g., $CD4^+$ $CD25^+$ $CD127^{low}$ can define the presence of human Tregs, but also of activated effector T cells).

In addition, appropriate product characterization with respect to the interactions between MSCs and other immune cells should be determined, as this justifies that the proposed potency assay reflects relevant biological properties. Several useful attention points (including MSC activation status, responder cell types, read-outs and so on) for the assessment of MSC immune regulatory properties have been provided by ISCT working groups [17]. Although meant for determination of MSC effects on T cells, the principles stated are also applicable to effects on other immune cells, although the activation stimuli and read-outs of the assays may be different.

Finally, if patients are treated (simultaneously) with other immunomodulatory agents, the effect of these agents should be taken into account in the potency assay when (significant) effect on the MSCs or responder cells is expected.

Immunomodulatory factors

Studies on effector molecules that play a main role in MSC activity are usually performed with animal models. However, there are significant species differences [85]. Mechanisms (both effector molecules as well as receptors) in other species are not directly comparable with humans and should therefore preferably not be the only base for the development of a potency assay. When cells from non-human species are used in a potency assay (e.g., as proposed by Nazarov *et al.* [44]), it should be demonstrated that the effects of the MSCs on these cells are comparable with the effects of the MSCs on human cells.

Inflammatory environment

MSCs are not constitutively immunosuppressive and require activation and further stimulation [32,37,63]. *In vitro* activation of MSCs can be achieved with various cytokines, including IFN-γ, TNF-α, IL-1α or IL-1β. And there are various factors that can mediate proper responder cell activation. Overstimulation of responder cells, however, could bring about artificial induction of specific intracellular pathways.

Because the inflammatory environment is crucial to enable MSCs to exert their immunosuppressive functions, dissecting the features of the microenvironment in characterization and pre-clinical studies is essential to understand the therapeutic effect of a MSC product [35]. Therefore, the choice of stimuli and the amount of cells (including their ratio) used in the *in vitro* potency assay is dependent on the *in vivo* environment at the site of inflammation [19]. In addition, the impact of cytokines (and other stimuli) used in cellular assays on the functionality of MSCs should be determined. For example, it is not uncommon to add exogenous IL-2 to stimulate T cell survival and proliferation, but this cytokine may also impact the suppressive function of MSCs [65].

However, it is acknowledged that it will be nearly impossible to determine the exact combination of factors, their concentrations and their timing of expression. As a consequence, it will be rather difficult to set up a MSC functionality assay that correlates with the *in vivo* situation because the combination and concentration of inflammatory mediators are critical in eliciting the immunosuppressive function of MSCs *in vivo* [37]. However, several good animal models of immunologic disorders exist and different *in vivo* and *in vitro* approaches can be applied to reveal the main factors involved in the pathogenesis and inflammation of specific immunorelevant diseases and provide guidance for the development of a relevant potency assay [86,87].

Timing

It should be shown that MSCs are capable of inhibiting already activated responder cells because the *in vivo* immune response is already on-going when MSC treatment will start. This would require an assay were T cells are activated prior to the addition of the MSCs. Moreover, the specific timing of MSC addition to the culture of activated responder cells should be optimized, as delayed addition of MSCs may impact the immunosuppressive capacity of these cells [68].

Irradiated MSCs

If MSCs are irradiated prior to administration to the patient, for potency characterization and release testing also irradiated MSCs should be used, although irradiation does not seem to influence the immunomodulatory capacity of MSCs [73].

Future perspectives and conclusion

The majority of MSC medicinal products in clinical development are indicated for GvHD or transplant rejection. Nevertheless, several MSC products are currently tested for treatment of chronic inflammation in autoimmune diseases, for example, systemic lupus erythematosus, systemic sclerosis, type 1 diabetes mellitus, multiple sclerosis, Crohn's disease and rheumatoid arthritis [2,19,21,32,88,89]. Although most potential MSC products for these indications are still in experimental or pre-clinical phase, it shows that

(application of) MSCs can be expected in all fields of immunotherapy.

Whatever their indication, every MSC product is considered unique, because the characteristics of these cells differ between donors (age and gender), tissue of origin, isolation and culture method, number of passages and further modulations [17,20]. Therefore, each MSC product will require its own characterization studies and quality control assays. Next to phenotypic characterization for identity and purity, regulatory authorities request a potency assay that tests for biological activity. Unfortunately, the MSC mechanisms of action are still not completely identified, resulting in the frequent use of potency assays based on surrogate markers or tests or a matrix of several complementary assays. This would, however, not prevent the production of an efficacious MSC product.

As discussed, most, if not all, assays have their shortcomings and some assays are preferred above others. Despite all uncertainties, T cell inhibition testing appears to still be the assay of choice for the analysis of MSC potency. Nevertheless, because potency assays will be assessed case-by-case, none of the mentioned tests are precluded in advance. Assays based on other MSC or responder cell properties can be appropriate, provided that the relevance of the proposed assay and read-out parameters is properly justified and substantiated with sufficient product-specific non-clinical and clinical data.

It is acknowledged that the MSC field will further evolve and continuous enhancement of quality control testing can be expected. It is also anticipated that improved standardized culture methods and procedures for the development of well-characterized (in-house) reference material will become available. Additionally, most studies cited here were performed *in vitro* or *ex vivo* and consequently more *in vivo* knowledge is needed before solid statements about MSC modulatory capacities for human immunotherapy can be made. The quest for appropriate potency assays will certainly contribute to this.

Acknowledgments

We thank Dr. Carla Herberts for useful comments and suggestions on the manuscript. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Disclosure of interests: The authors have no commercial, proprietary or financial interest in the products described in this article.

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