Mesenchymal Stromal Cells for Graft Versus Host Disease: Mechanism-Based Biomarkers

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The immunosuppressive activity of mesenchymal stromal cells (MSCs) in graft versus host disease (GvHD) is well-documented, but their therapeutic benefit is rather unpredictable. Prospective randomized clinical trials remain the only means to address MSC clinical efficacy. However, the imperfect understanding of MSC biological mechanisms has undermined patients’ stratification and the successful design of clinical studies. Furthermore, although MSC efficacy seems to be dependent on patient-associated factors, the role of patients’ signature to predict and/or monitor clinical outcomes remains poorly elucidated. The analysis of GvHD patient serum has identified a set of molecules that are associated with high mortality. However, despite their importance in defining GvHD severity, their role in predicting or monitoring response to MSCs has not been confirmed. A new perspective on the use of MSCs for GvHD has been prompted by the recent findings that MSCs are actively induced to undergo apoptosis by recipient cytotoxic cells and that this process is essential to initiate MSC-induced immunosuppression. This discovery has not only reconciled the conundrum between MSC efficacy and their lack of engraftment, but also highlighted the determinant role of the patient in promoting and delivering MSC immunosuppression. In this review we will revisit the extensive use of MSCs for the treatment of GvHD and will elaborate on the need that future clinical trials must depend on mechanistic approaches that facilitate the development of robust and consistent assays to stratify patients and monitor clinical outcomes.

Keywords: mesenchymal stromal cell, graft versus host disease, biomarker, apoptosis, efferocytosis, extracellular vesicles

A BRIEF HISTORY OF MSCs

Mesenchymal stromal cells (MSCs) are typically described as a highly heterogeneous population of stem and progenitor cells selected and expanded in vitro as unfractionated fibroblastic-like and plastic-adherent cells (1). This population was first described in the early 1970s by Friedenstein and colleagues who isolated from the bone marrow (BM) of guinea-pigs and mice a group of fibroblastoid cells able to differentiate into adipocytes, chondrocytes and osteocytes and to reconstitute the microenvironment for the culture of hematopoietic stem cells (HSC) (2, 3). These cells were later identified in human tissues (4) and referred to as mesenchymal stromal cells (MSCs) (5).
Since their first description, cells with analogous characteristics have been successfully isolated and expanded from many other tissues (6), such as placenta (7), umbilical cord (UC) (8), adipose tissue (AT) (9), and dental pulp (10). MSC identification relies on the combined expression of CD73, CD90, CD105, CD71, CD44, CD106, and the lack of hematopoietic and endothelial markers (CD34, CD45, CD11b, CD14, and CD31) (11). The definition of MSCs features a substantial overlap with the traditional concept of other more mature stromal cells, such as fibroblasts (12–14), making it plausible to consider that these are equivalent or related cell types.

MSC heterogeneity has been described within the same species (15), tissue preparations (16, 17) and even on same donor isolations. As observed by Mets and Verdonk, during MSC sub-cultivation, younger passages were characterized by higher rates of plasticity and proliferation compared with older passages (18). Yang et al. (19) also described gradual loss of the typical fibroblast-like spindle shape, decreased expression of the adhesion molecule CD146 and genetic instability in human MSCs under increasing in-vitro passages. Despite their heterogeneity, MSCs have been largely employed in experimental cell-based therapies for treating human diseases. Historically, the lack of the expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules (CD40, CD40L, CD80, and CD86), associated with low levels of MHC class I on MSC surface (20, 21), initially introduced the idea of a population of “immune-privileged” cells which could be widely used beyond MHC- compatibility restrictions (22), and this consideration greatly ignited the enthusiasm around MSCs as therapeutic tools.

The possibility that MSCs might be devised as therapeutically effective cellular products mainly derived from studies describing MSC ability to improve tissue healing and regeneration (23, 24) and to alter host immune responses by suppressing inflammation (22, 25–28). It has not been fully elucidated how healing and immune suppressive MSC properties are intertwined. However, they are not mutually exclusive or completely independent as tissue regeneration requires resolution of injury-associated inflammation. In this review, the multipotency of MSCs will not be further discussed [reviewed in Bianco et al., (29) and Caplan (30)]. Conversely, immunosuppression mediated by MSCs will be extensively examined.

It is widely accepted that MSC immunosuppressive properties are not constitutive. Instead, their immune regulation depends on a process of “licensing” which needs to be acquired in an inflammatory microenvironment. This concept finds support not only in vitro but also in preclinical models of graft versus host disease (GvHD), whereby MSC therapeutic activity could be obtained only when MSCs were infused in the presence of a specific inflammatory milieu (31). Accordingly, MSCs were effective in reducing GvHD signs only when multiple infusions were administered after transplant but not when single dose was co-infused with HSC transplantation (HSCT) (32). These experimental observations have been strongly supported by a meta-analysis recently performed by Wang and collaborators (33). In this work, the authors included 6 randomized clinical trials enrolling 365 patients. MSCs were infused at different time points from HSCT (within 24 h, at a median time of 28 days, or with multiple infusions at different time points). The analysis showed that infusion of MSCs significantly reduced the incidence of chronic GvHD (cGvHD) and there was a trend of longer overall survival in MSC-treated patients (33). Importantly, the meta-analysis on different sub-groups demonstrated that these favorable outcomes were significantly associated with late MSC administrations, thus supporting a more effective role of MSCs when licensed by a specific microenvironment developed after HSCT.

Once licensed, MSCs are able to mediate potent immunoregulatory effects through diverse modes of action on a variety of cell types, involving both the adaptive and innate immunity. The immunomodulatory repertoire induced by primed MSCs includes factors such as indoleamine 2,3-dioxygenase (IDO) (34–36), prostaglandin E2 (PGE2) (28, 34), heme oxygenase-1 (HO-1) (37), transforming growth factor beta (TGF-β), IL-10 (38), human leukocyte antigen-G5(HLA-G5) (39), leukemia inhibitory factor (LIF) (40), and galectin 1, 3, and 9 (41–43). IDO, PGE2 and HO-1 can directly induce metabolic reprogramming on activated T lymphocytes, reducing their proliferation rates, cytokine production and cytotoxic activity (28, 34, 37, 44). Likewise, MSCs suppress the proliferation and modulate the cytokine production of activated natural killer (NK) cells (45, 46) through the action of IDO and PGE2 (28, 34, 36, 47). Furthermore, MSCs can inhibit B-cell activation, proliferation and IgG secretion both in vitro and in vivo (44, 47) in a soluble-factor dependent manner (48). In addition, MSCs can dampen the activation of effector immune cells via cell-cell interactions through the association of the programmed death 1 and its ligand (PD-1/PDL1) (49, 50) and HLA-G1 (51).

Reprogramming of the host immune cells is another mean of MSC immunomodulation, especially in vivo. MSCs can recruit monocytes to the site of inflammation by the secretion of chemokine ligand 2 (CCL2) (52). In a heart injury model, MSCs reduced the number of pro-inflammatory monocytes, while increased those with anti-inflammatory phenotype (53). Moreover, in the presence of colony stimulating factor 1 (CSF-1/M-CSF), MSCs can promote monocyte differentiation into macrophages with upregulated expression of CD206, IL-10, and TGF-β and improve phagocytic efficiency, which suggests the characteristics of M2 macrophage differentiation (54). Likewise, bone marrow progenitors are induced to differentiate into a population of CD11b+ myeloid cells with potent suppressive activity in the presence of MSCs. Such differentiation is mediated by nitric oxide synthase-2 and these MSC-educated CD11b+ cells accelerate hematopoietic reconstitution in bone marrow transplant recipients (55).

MSCs can also inhibit monocyte differentiation into dendritic cells (DCs) and skew them into a tolerogenic profile via reducing their expression of HLA-DR, CD1a, CD80, and CD83 as well as down-regulating their IL12 secretion (56–59). Moreover, effects of MSCs on regulatory T cells (Treg) expansion have also been documented in many inflammatory conditions (60–62). MSCs induce differentiation of functional Treg through TGF-β, HLA-G5, IDO, and PGE2 (39, 59, 63). Remarkably, MSCs can further favor Treg expansion in vitro indirectly by inhibiting DC maturation, CD8 T cells, and NK cells expansion (59, 64).
The up-stream mechanism of MSC licensing has been a puzzle for decades in MSC research. Pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1α, or IL-1β have been extensively reported on MSC activation in vitro. These molecules are predominately secreted by activated monocytes or T cells, and subsequently trigger the production of immunosuppressive molecules in MSCs (35, 36, 65, 66). Blockage on either these soluble factors or cell-contact pathways successfully undermined MSC immunomodulatory effects. Yet, generally none of these molecules taken alone is sufficient to account for MSC suppressive function which in fact seems to be the result of a synergistic combination of more factors. Therefore, how these molecules are intertwined in vivo and the degree of redundancy of their effects on MSC licensing warrants further investigation.

CURRENT CHALLENGES ASSOCIATED WITH THE USE OF MSCs FOR THE TREATMENT OF GvHD

MSCs Use as a Therapeutic Tool

The characteristics described in the previous paragraph elicited the interest in MSCs, considered as promising therapeutic tools to control aberrant inflammatory responses. As shown in Figure 1, consultation of the public registry of clinical trials at the U.S. National Institute of Health database (at ClinicalTrials.gov) shows a continuous increase of the number of new studies involving MSCs for the treatment/prophylaxes of immune-mediated diseases which were registered between 2004 and 2010, with at least 6 new registrations thereafter.

The focus of the use of MSCs as cell-therapy products has been mainly focused on two aspects: (1) the use of MSCs to exert peripheral tolerance in contexts whereby this tolerance was altered after the use of MSCs (i.e., usefulness of MSCs as prophylaxes), and (2) the use of MSCs when an inflammatory or autoimmune response was already established before MSC infusion (i.e., MSC use as specific therapy to restore peripheral tolerance). Aim of this review is to focus on the use of MSCs after HSCT and in GvHD patients.

MSCs for the Treatment of GvHD

GvHD is a life-threatening complication of allogeneic HSCT, and currently represents one of the major factors limiting the success of this potentially curative option for hematological malignancies (67, 68). GvHD has been classified into acute (aGvHD) and cGvHD (69, 70). Currently, there is no standardized treatment for patients with aGvHD who do not respond to steroids, and their prognosis is still very poor, with overall survival inferior to 20% at 2 years (71). The interest in MSCs for the treatment of aGvHD has sparked remarkably since the very encouraging results published in 2008 by the European Group for Blood and Marrow Transplantation Developmental Committee (72), with 30 out of 55 patients with steroid resistant aGvHD showing complete response to MSCs. Importantly, these responding patients had 55% overall survival at 2 years. To date (last analysis in September 2019), at least one new clinical trial involving the use of MSCs to mitigate GvHD has been registered every year at ClinicalTrial.gov with a peak of 5 different studies started in 2015 (Figure 1). A systematic search of the published manuscripts in peer-reviewed journals has identified 14 studies (72–85), both retrospective and interventional, with more than 30 patients enrolled. In these studies, all aGvHD patients were steroid-resistant. Only one study used MSCs as first line treatment in...
TABLE 1 | Clinical studies with MSCs used in aGvHD.

| Publication                  | Patients | MSC infusion | Outcome |
|------------------------------|----------|--------------|---------|
|                              | N        | Median age (range) | Dose (x10^6/Kg) | Median (range) | CR (%) | PR (%) | NR (%) |
| LeBlanc et al. (72)          | 55       | 22 (0.5–64)     | 0.40–9.00       | 2–1 (5)       | 54     | 16     | 29     |
| Ball et al. (73)             | 37       | 7 (0.7–18)      | 0.90–3.00       | 2–1 (13)      | 65     | 22     | 13     |
| Kurtzberg et al. (74)        | 75       | 8 (0.2–17)      | 2.00            | NRe (8–12)    | NRe    | NRe    | NRe    |
| TelBoom et al. (75)          | 48       | 44.9 (1.3–68.9) | 1.80 (0.90–2.50) | 3–1 (4)       | 25     | 50     | 25     |
| Kebriaei et al. (76)         | 31       | 52 (34–67)      | 2.00–8.00       | 2–1 (6)       | 77     | 16     | 7      |
| Erbey et al. (77)            | 33       | 7 (3–18)        | 0.50–2.80       | 2–1 (4)       | 54     | 21     | 25     |
| Servais et al. (78)          | 33       | 58 (5–69)       | NRe (1.00–4.00) | 1–1 (2)       | 22     | 41     | 37     |
| vonDalcowski et al. (79)     | 58       | 55 (19–71)      | 0.99 (0.45–2.08) | 2–1 (8)       | 9      | 38     | 53     |
| Dotoli et al. (80)           | 46       | 28 (1–72)       | 6.81 (0.98–29.78)** | 3–1 (7)     | 7      | 43     | 50     |
| Bader et al. (81)            | 69       | 8.2 (6 mo–18)   | 45.5 (18.9–65.5) | NRe (1.00–2.00) | NRe    | NRe    | NRe    |
| Introna et al. (82)          | 37       | 27.8 (1–65)     | NRe (0.80–3.10) | NRe (2–11)   | 30     | 43     | 27     |
| Fernandez-Mazqueta et al. (83)| 33     | 46 (18–61)     | 1.06 (0.66–1.76) | 1.06 (0.66–1.76) | 34     | 50     | 16     |
| Resnick et al. (84)          | 50       | 19 (1–69)       | 1.00 (0.3–3.10) | NRe (1–4)    | 34     | 32     | 34     |
| Galleu et al. (85)           | 60       | 40 (4 mo–68)    | 2.60 (0.60–15.60) | 1–1 (4)     | 2      | 52     | 46     |

CR, Complete Response; NRe, Not reported; NR, No Response; PR, Partial Response; *3%, no data available at day 28; **cumulative dose.

association with steroids (76). Both pediatric and adult patients were treated with age ranging from 2 months to 72 years. It is not possible to directly compare these studies in terms of efficacy due to the heterogeneity of the patients enrolled. However, results seem to be very encouraging. Indeed, as summarized in Table 1, overall response rates ranged from 47 to 93% even though patients were mostly resistant to multiple lines of treatments. Notably, the use of MHC-matched, haploidentical or third-party MSC donors does not have any impact on patient outcomes (72–85).

MSCs could successfully be expanded from disparate tissues, spanning from BM (72–82, 84), UC (86, 87), AT (88, 89), or placenta (90). BM has been the first MSC source ever described and the most frequently deployed thus far. However, the origin of MSCs does not seem to affect their anti-proliferative and immunological properties in vitro (91). Furthermore, despite the small number of patients treated with UC (86, 87), AT (88, 89) or placenta (90), similar response rates were reported when compared to the outcomes obtained when BM-MSCs were used (Table 1), thus supporting the role of these sources as valid alternatives for clinical-grade MSC production. In fact, UC and AT may be considered as more “affordable” alternative sources in terms of manufacturing logistics and costs compared to BM. Obtaining MSCs from UC or AT has important advantages. First, the invasive BM harvest procedure, associated with (minimal) risk for donors, can be spared. Secondly, both UC- and AT-MSCs can be obtained from tissues which are currently otherwise discarded and also from samples previously frozen before isolation (this at least it has been described in UC-MSCs) (92, 93). Third, they have higher proliferative capacity and longer life-span in vitro with higher cells yielded per expansion (94, 95).

MSC therapeutic activity has been tested also in cGvHD, albeit the experience in this setting is more limited than in aGvHD. Most studies reported the treatment of only few patients, and they should be considered as case reports. Results were in fact variable, with overall responses ranging from 0% (82, 96) to more than 50% of the patients treated (97–100). More promising are the results obtained from larger groups of patients and reported in three different studies. In two of these studies, a total of 57 steroid-refractory cGvHD were treated with 1–5 infusions of BM-MSCs. The median time to response varied between 3 and 24 months after the first MSC infusion (101, 102). Notably, 26% of the patients treated could wean immunosuppressive therapy until complete discontinuation in one of the studies (102). Recently, 14 patients with moderate to severe cGvHD were prospectively treated with one infusion of AT-MSCs as first-line treatment in association with steroids and cyclosporine (103). In total, 13 patients could be evaluable, since 1 patient withdrew participation consent. Ten patients achieved a response at 56 weeks [8 complete response [CR] and 2 partial response [PR]], all stopped steroids and were alive at the end of the study. Conversely, of the 3 non-responding patients, none was alive and the cause of death was progressive cGvHD (103).

MSCs for the Improvement of HSCT and as Prophylaxis of GvHD

MSCs have been demonstrated to enhance haematopoietic engraftment and hematological recovery after both autologous (104) and allogenic (105–107) HSCT when administered at the time of transplant. This property may become crucial in situations in which, due to damage of the BM niche after conditioning regimens for HSCT, haematopoietic recovery may be severely delayed. Koc et al. (104) were the first to report improvement of haematopoietic engraftment when autologous BM-MSCs were co-transplanted with HSCT. These findings, along with positive results from preclinical models whereby MSCs were able to delay the onset of GvHD (108, 109), prompted investigators to assess whether MSCs could be used for the improvement of HSCT engraftment and prophylactically
to decrease the frequency of GvHD when co-administered with the transplant. The ability of MSCs of improving HSCT engraftment, or preventing graft failure, seemed to be confirmed in some studies (106, 107, 110–113). However, absence of any improvement has also been reported (114, 115). Recently, a comprehensive meta-analysis carried out by Kallekleiv et al. (116) determined the potential benefits of MSCs when co-administered with allogenic HSCT within a range of 24 h (before or after the transplant). The study included a total of 309 patients enrolled in 9 controlled trials performed until May 2015, thereof 3 randomized and 6 non-randomized studies. The analysis suggests that MSCs do not have any beneficial effects in terms of facilitation of engraftment or either aGvHD nor cGvHD prevention (116). Important limitation of this meta-analysis relates to the small sample sizes of the studies included and their weak designs, thus results should be interpreted with caution.

Taken together, these data suggest that, while MSC use is safe, the efficacy of this treatment as tool to promote HSCT engraftment or GvHD prophylaxis should not be routinely supported. Factors which may play a role in influencing the activity of MSCs include the concomitant therapy, the underlying disease or the conditioning regimen. By modifying the inflammatory milieu of the patient, these components may affect the MSC “licensing” and hamper their immunomodulatory capacity to reset the haematopoietic niche.

**MSC Biomarkers for GvHD: An Unmet Need**

In the previous paragraph, we have reported the very encouraging results when MSCs are used for the treatment of aGvHD. However, the only randomized phase III trials, sponsored by Osiris Therapeutics (NCT00562497 and NCT00366145) and making use of commercially available MSCs (Prochymal), missed their endpoints and failed to demonstrate efficacy of MSCs. Nonetheless, this failure was only announced by press-release and results were never published in peer-reviewed manuscripts. To make them more difficult to interpret, the publicly available results (published in abstract forms only) did demonstrate the efficacy of MSC treatment in specific sub-categories of patients with improvements in response rates in pediatric patients (117) or patients with gut or liver GvHD (118).

These results and the contrast with the outcomes reported in most phase II studies raised many questions on the possible causes of this failure (119). Furthermore, it drove to question the very same utility of MSCs as part of the available treatments of GvHD, as highlighted by the recent clinical commissioning policy on GvHD treatments published by NHS England, which concluded that there was not enough evidence for supporting the use of MSCs in GvHD patients (120). It is conceivable that to definitely and robustly assess the role of MSCs in GvHD therapeutic armamentarium, we need prospective phase III trials whose design needs to be guided by potency assays or biomarkers able to effectively stratify patients and predict clinical responses.

A biomarker (or biological marker) is a parameter that can be objectively measured or evaluated to indicate a biological process, pathogenic processes, or pharmacologic responses to a therapeutic intervention (121). In the regard of MSC therapy in aGvHD, ideal MSC biomarkers can be served as a prognostic tool to (1) forecast the clinical outcome, or (2) predict the clinical response, or (3) monitor the efficacy of MSC therapy among a variety of aGvHD patients.

There have been two major approaches to predict or monitor the therapeutic effects of MSC in GvHD. The first approach has been to apply a panel of GvHD biomarkers, which are molecules related to the tissue damage during the pathogenesis of aGvHD (122). They were first identified to provide diagnostic and prognostic information on GvHD independently of the clinical symptoms (122). The initial panel included the plasma level of interleukin 2 receptor subunit α (IL-2Rα), tumor necrosis factor receptor 1 (TNFR1), interleukin-8 (IL-8), and hepatocyte growth factor (HGF). Subsequently, the same research group included two organ–specific biomarkers, which are regenerating islet-derived 3α (Reg3α) (123) and elafin (124), specific for gastrointestinal and skin GvHD, respectively. Several studies in aGvHD have reported the change of these biomarker after the MSC infusion and their correlation with the MSC response. For instance, Dander et al. (125) found a decrease of plasma TNFRI, IL-2Rα, and elafin in those patients who responded to MSCs but not in the non-responders. At the same year, von Bahr et al. (126) reported similar decline of serum IL-2Rα in GvHD patients after MSC infusion, although they did not compare the change of IL-2Rα between responders and non-responders. Later, Yin et al. (127) found a fall of Reg3α and cytokeratin fragment 18 (CK18), another tissue damage biomarker in liver and intestinal GvHD, in MSC responders (128). However, discrepancies have also been reported. In contrast to Yin et al. (129) another study indicated that Reg3α and IL-2Rα were not correlated with the response to MSCs in aGvHD patients. Furthermore, in a phase II study, there was no correlation of any GvHD biomarkers with the clinical response following MSC treatment in aGvHD patients (75), raising questions on the reliability of these GvHD biomarkers in monitoring MSC efficacy.

The second approach has been to monitor some effector molecules or cellular pathways reported as mediators of MSC immunosuppression in vitro and in pre-clinical studies. Dander et al. (125) reported an increase in the proportion of Treg compared to Th1 and Th17 cells after MSC treatment in the responders, while opposite results were found in the non-responders. However, another study did not find any increase of the Treg population in both responders and non-responders. Moreover, both the numbers and functions of CD4 and CD8 T cells also remained unchanged. The only significant difference between responders and non-responders was the proportion of immature DCs which was increased among the responders following MSC infusion (75). Similar ambiguity was also noticed in the effector molecules which have been described in MSC immunosuppression in vitro. For instance, no changes of the serum level of IL-6 or HLA-G was detected in patients after receiving MSCs regardless of the clinical response (126). Importantly, the same study also found that MSC immunosuppressive potency, measured as anti-proliferative activity against T cells after stimulation in vitro, did not correlate with MSC clinical efficacy in vivo. This observation
indicates that the role of this in vitro potency assay was not optimal to predict the clinical efficacy of MSCs in aGvHD patients (126).

Albeit not strikingly biomarkers by definition, there have been several associative factors which can seemingly influence MSC responses among aGvHD patients. Dosage of MSC infusion, age of MSC recipients and organ involvement have been reported as affecting responses in aGvHD patients (72, 77, 78, 82, 84, 85, 130). Briefly, patients who received higher MSC doses (78, 85), younger patients (72, 82, 84, 85), or patients with gut or/and skin involvement seem to achieve a better response to MSCs (77, 85, 130). However, these results have not been confirmed in other studies (73, 79, 131), and should be considered with caution. These discrepancies highlight the weak reliability of these associative factors when used as predictors of response. Furthermore, the biology underlying these clinical observations is still unknown. Nevertheless, they unquestionably stress the importance of the patient as crucial player in the response.

The Paradox of MSC Immunosuppression Models (in vitro vs. in vivo)
The unsatisfactory ability to predict or monitor clinical responses to MSCs by the panel of molecules described in the previous paragraph can be attributed to two main reasons. First, the proposed GvHD biomarkers (e.g., Reg3a and Elafin) (75, 125, 128, 132) appear to be reliable sensors of the severity of the disease and tissue damage but they lack any mechanistic rationale regarding the immunosuppressive activity of MSCs and their licensing. It is then arguable that their impact on MSC clinical response may be minimal. However, if variations of their values among responders and non-responders are consistently found in larger studies, they will acquire a more defined role in the monitoring of the GvHD after MSC treatment.

Second, the immunosuppressive effector pathways that have been extensively and elegantly characterized as crucial in models of MSC immunosuppression in vitro (75, 125, 126, 128, 132) have not been demonstrated to have a reproducible role in predicting MSC therapeutic activity in vivo. The impossibility to exploit these molecules as biomarkers should also be taken into account. Although it is relatively easy to determine and monitor these soluble molecules in vitro, our ability to measure them in vivo might be jeopardized by their restricted range of action and limited bioavailability over time. The correct timing to assess these effectors may also differ significantly based on the nature of studies (in vitro vs. in vivo), with a further layer of complexity associated with difference in metabolism secondary to patient age, disease severity, co-morbidities, or use of concomitant treatments.

At the state of the art, our well-established in vitro models of MSC immunosuppression are not fully elucidated for the development of robust biomarkers as predictors of clinical response in GvHD patients treated with MSCs. It appears that a better understanding of the mechanisms underlying MSC-mediated immunosuppression in vitro is therefore fundamental to provide novel perspectives and mechanistical platforms as starting points for the development of reliable biomarkers.

ROLE OF MSCs UNDERGOING APOPTOSIS TO DELIVER IMMUNOSUPPRESSION IN VIVO
MSCs Undergoing Apoptosis in vivo to Induce Immunosuppression
One major unresolved challenge which undermines the progress in our understanding of MSC immunosuppression in vivo is that the vast majority of infused MSCs become undetectable a few hours after transiently residing in the lungs (133, 134). Nevertheless, MSCs appear to maintain their ability to deliver therapeutic activities and engage with other regulatory cells like T-reg and macrophages. It is clear that our current in vitro models of MSC immunosuppression are still lacking some important aspects and cannot reconcile the paradox of the absence of engraftment and immunosuppressive functions (44, 135–137).

Starting from these observations, we tested the hypothesis that the lack of MSC engraftment might be due to cell death after infusion. In our experimental model of aGvHD, we have demonstrated that MSCs undergo extensive caspase activation and apoptosis after infusion in the presence of cytotoxic cells, and that this is a requirement for their immunosuppressive function (138). This apoptosis is mediated by both CD8 and NK cells and is not MHC-restricted. After MSC apoptosis, phagocytic cells are also required to engulf apoptotic MSCs and produce IDO which in turn triggers immunosuppression (138). These findings are in line with previous studies, whereby activated but not resting NK cells were able to lyse MSCs in vitro (46), or MSCs were cleared in vivo by deployment of different recipient-dependent reactions (139–143). Notably, these data provide a completely novel perspective which undermines the so-called “immune-privileged” status of MSCs. Conversely, by demonstrating the instrumental role of in vivo MSC apoptosis in delivering immunosuppression after infusion, they reconcile the role of the observed MSC rejection in vivo (144) in the context of MSC immunosuppressive functions across MHC barrier (72, 145) and highlight the capacity of apoptotic MSCs to modulate immune responses (146–149).

MSC Apoptosis Provides a Predictive Biomarker Selecting MSC Responders in GvHD
The observation that MSC apoptosis requires and is induced by cytotoxic granules in a mouse model of aGvHD led us to investigate the role of cytotoxic immune cells against MSCs also in human patients. We found that the cytotoxic activity against MSCs can also be detected in the PBMCs of GvHD patients. More importantly, our data show that patients displaying high cytotoxicity respond to MSC therapy, whilst those with low or absent cytotoxic activity do not improve following MSC infusion (138). These data have now been confirmed in an extended cohort of patients and the cytotoxic assay has been found to predict clinical responses with high sensitivity and specificity (Galleu A et al. Oral presentation, Abstract S252, EHA).
2020). It is important to point out that the limited number of patients analyzed warrants further validation in a prospective clinical study.

Currently, we do not know whether the cells mediating this cytotoxicity are derived from the donor of HSCT or from the recipient. Furthermore, it cannot be excluded that patients who have very poor reconstitution after HSCT with both CD8 and NK cells may have a hampered capacity to kill MSCs and then reduced likelihood to respond to MSC treatment. However, neither the absolute numbers nor the frequencies of CD8 and NK cells seem to have a role in predicting the response to MSCs (75), as supported by our observation that there is no difference between CD8 or NK cell percentages between responders and non-responders (138). It is likely that only a better characterization of the phenotype of the cytotoxic cells mediating MSC apoptosis will enable us to identify the actual subpopulation of cells eventually responsible of this apoptosis and to develop a quantitative and more approachable assay for use in a routine pathology laboratory.

Despite these limitations, this predictive biomarker represents a paradigm shift in MSC therapeutics. Its strength relies on the fact that the assay is supported by mechanistic insights. Furthermore, an important consequence of these observations is that, although MSCs remain the necessary starting point for therapeutic immunosuppression, patient-derived cells play a crucial role in delivering such an immunosuppression. This new perspective, in line with clinical data whereby MSCs from the same donor can give different responses in different patients (72–74, 76, 78, 79, 150), may significantly affect the Research and Development sector of MSC manufacturing. In the last decades, much efforts have been spent on the identification of the most clinically effective MSC preparations. Several strategies have been proposed, including the selection of MSCs based on biological parameters such as the magnitude of IDO synthesis (151) or the intracellular levels of the transcription factor TWIST1 (152). Conversely, other groups suggested to overcome the intrinsic variability among MSC batches by generating MSCs from pooled BM-MNCs of multiple third-party donors (153). It is not clear whether different MSCs exhibit similar or different capacity to undergo apoptosis. Further studies are needed to verify whether MSCs from different sources, administered after thawing or from fresh cultures, expanded in selected conditions, or differentially sorted based on specific features, have different susceptibility to undergo apoptosis. In this perspective, the cytotoxic assay may be devised as a tool for standardization of MSC manufacturing by select specific thresholds of killing used as product specification. Such an assay would also address the unmet need for a potency assay as a guideline for Regulatory Authority requirements (154) to implement quality control of manufactured MSCs. Thus far, most potency assays are designed with the aim to identify or select the “most immunosuppressive” MSC batches (155, 156), but they are exclusively based on MSC in vitro properties. By measuring their susceptibility to undergo apoptosis when exposed to cytotoxic cells, the cytotoxic assay would possibly identify “the most fit MSCs” which will deliver their therapeutic activity once administered to patients able to induce their apoptosis.

MSC Apoptosis Provides a Monitoring Biomarker Evaluating MSC Immunological Effects in GvHD

The role of MSC apoptosis in vivo not only provides clinicians a powerful prognostic tool to predict patient responses to MSC treatment (138), it also paves the way for the development of potential tool to monitor the immunological effects after MSC infusion. The ground for this approach will be centered around the concept of the reprogramming of myeloid cells in the hosts following MSC apoptosis and efferocytosis. It has been well-documented that robust immune suppression and tolerance is mediated by myeloid cells (monocytes and dendritic cells in particular) following efferocytosis of apoptotic cells. These effects can be mediated by TGF-β (157, 158), IDO (147), IL-10 (159), or COX2/PGE2 (160). The field of dying MSCs has only begun to unveil their immunomodulation in certain models (161) and remains largely unexplored. However, it is conceivable that some of these factors might emerge as valuable biomarkers when further investigated.

In this perspective, the latest findings from our group seems to corroborate this idea. Indeed, we have demonstrated that efferocytosis of apoptotic MSCs endows monocytes with antiproliferative activity against T cells (162). These monocytes upregulated several immunosuppressive molecules, including metabolic enzymes IDO and COX2, immune checkpoint ligand PDL1 as well as soluble factors PGE2 (enzymatic product of COX2) and IL-10. Of note, the activity of COX2/PGE2 within the monocytes is in fact the key to determine the downstream expression of IDO, PDL1, and IL-10 as well as the monocyte inhibitory effects against T-cells. Most importantly, in a cohort of steroid-refractory aGvHD patients, the increase of serum PGE2 after MSC treatment is significantly higher in the responders compared to the non-responders. Hence, we suggest that the serum level of PGE2 can be evaluated as a biomarker for the monitoring of the immunological effects of MSCs in aGvHD patients receiving MSC treatment. PGE2 can be easily measured with the current biochemical methods such as ELISA, a rapid protocol with high sensitivity, specificity and reproducibility. With a reliable MSC monitoring biomarker, clinicians can be benefited from an early predictor of treatment failure, thus promptly pursuing alternative treatments before the assessment of a response. Furthermore, this tool can be devised to optimize the MSC dosage or design a combinational regimen to improve the clinical efficacy of MSC therapy.

POTENTIAL OF MSC EXTRACELLULAR VESICLES (EVs) AS BIOMARKERS IN GvHD

Besides the long-term notion about the importance of growth factors and cytokines as a part of the cell communication, the concept that cells also secrete large amounts of extracellular vesicles (EVs) as potential mediators is relatively new (163, 164). EVs are spherical structures limited by a lipid bilayer, which contains hydrophilic soluble components such as proteins, small and large RNA and DNA. There are different types of secreted EVs that have distinct structural and biochemical
properties depending on their intracellular site of origin (165). Microvesicles and apoptotic bodies have been described as large EVs (>100 nm diameter) and can be formed at the plasma membrane by direct budding into the extracellular space. Smaller vesicles referred to as exosomes (around 100 nm diameter) are originated in multivesicular endosomes, subsequently secreted by fusion of these compartments with the plasma membrane (166).

The interest in EVs has progressively grown due to the discovery of their functional content, and the knowledge that the different EV subtypes contain molecules derived from different cellular compartments. Omics studies revealed that exosomes contain proteins originally located in the endosomes and microvesicles from cytosol and plasma membrane (167, 168). Apoptotic bodies, on the other hand, can contain molecules from the nucleus, endoplasmic reticulum or Golgi (169). Such a selectivity confirms that their cargos are not random, as might be in the case of cell debris. Instead, EVs contain a set of well-characterized and evolutionarily conserved proteins including the protein family of tetraspanins (CD63, CD81, and CD9) as well as Alix and TSG101, which have been used as EV markers. Also, they contain a set of molecules that varies according to different physiology, therefore understanding the EV cargo modifications, for instance during inflammation, may provide valuable insights into the prediction and/or monitoring of pathological processes (170).

EVs have been purified from many types of cell culture and are believed to be released from most, if not all, somatic cells, either constitutively or upon activation. Hence, they can be found in all different biological fluids such as plasma, serum, saliva and urine. Due to this specific content, EVs have been proposed as suitable biomarkers for various conditions (166). For instance, the use of EVs as a biomarker in allogenic transplantation context has been extensively investigated (171). In the study from Gunasekaran et al. (172) in lung transplantation, the detection of graft-derived exosomes preceded clinical diagnosis of graft rejection, suggesting that they could serve as a method to predict chronic rejection and adjust patient treatment accordingly. The predictive use of EVs as a biomarker was also suggested by Zhang et al. (173) In their study, they identified a panel of mRNAs (gp130, SH2D1B, TNFα, and CCL4) present in plasma EVs that could be used to predict on-going and/or imminent antibody-mediated rejection in kidney transplants. Other researchers found that they could correlate plasma and urinary EV content with graft rejection and its severity in renal (174–176) and lung transplanted patients (177). These results have shown that monitoring EVs and their cargos in patients might represent a promising non-invasive method to evaluate the status of allografts and the type and stage of rejection.

MSCs are well-characterized producers of a wide range of EVs with different cargos. The presence of selected miRNAs within MSC-derived EVs has been first described by Collino et al. (178). In their studies, they found that some of the miRNAs were present in both the EVs and the original cells. The similarities between cells and EVs were further confirmed by Kim et al. (179), when they conducted a study characterizing the protein content of BM-MSC-derived EVs and revealed their overlaps in surface markers, signaling molecules, cell adhesion molecules and additional MSC antigens. These data suggest MSC-derived EVs as potent mediators of intercellular communication locally and systemically.

EVs released from licensed MSCs have different composition and probably roles, when compared to those released by resting MSCs. Several studies have recently reported a significant variation on the EV content depending on the extracellular microenvironment priming the MSCs (180–183). Although the characterization of EVs released by the apoptotic MSCs is still under investigation, it is well-documented that apoptotic cells can produce a range of EVs and apoptotic bodies with different cargos that influences their microenvironment (169, 184, 185). In this regard, we can characterize the MSC-derived EVs to monitor their licensing process and/or the process of apoptosis. Furthermore, they might reveal the immunological effects of MSCs. Therefore, monitoring EVs isolated from the circulation of patients receiving MSCs holds a promising non-invasive method to evaluate the clinical efficacy of the treatment. Lastly, the analysis of EV content over time could also give hints of MSC kinetics, allowing to the adjustment of MSC administration accordingly.

**CONCLUSIONS**

MSC immunobiology makes them ideal candidates for their use in cellular therapy in several immune mediated diseases, including GvHD. After thousands of infusions, the most convincing conclusion is that MSCs are well-tolerated and safe for patients. Major infectious events, secondary neoplasms, or malignancy relapse do not seem to increase after MSC therapy (33, 186). However, available data on MSC use in GvHD treatment represent the paradigm of the limitations of our current use of MSCs in most clinical applications. It is unquestionable that patients who responded to MSCs exhibit longer overall survivals than the non-responders (72–76, 78, 85). Importantly, this is a consistent finding across heterogeneous cohorts of patients (Table 1). Nonetheless, there is not definitive and proved evidence of efficacy and responses are unpredictable.

The furious arguments ignited on the legitimacy of the use of MSCs in GvHD in the last few years highlights the unmet need to better understand how to improve the durability and the rates of responses to MSCs. We believe that only an in-depth understanding of the reasons behind clinical responses represents the necessary milestone for the design of the next generation of clinical trials in MSCs. Their success will undoubtedly route on our ability to identify the effective instruments (namely biomarkers and functional assays) that help us to predict clinical responses, guide us in selecting the best patient candidates, and ideally provide information as early predictors of treatment failure. Our ability to select only “fit patients” will be crucial in terms of sustainability of the costs of the MSC treatment and of a better management of limited resources, especially in the case of universal health care systems.
FIGURE 2 | MSC immunomodulation depends on the interaction with the host. Schematic representation of MSC mediated immunosuppression after infusion. 1: After infusion, MSCs interact with the cytotoxic granules produced by CD8 T cells and NK cells of MSC recipient. 2: MSCs are induced to undergo apoptosis. 3: Apoptotic MSCs are cleared from the circulation by the mononuclear phagocyte system. After efferocytosis, phagocytic cells of MSC recipient are induced to produce PGE2 and IDO which are the final mediator of MSC immunosuppression. Importantly, while the cytotoxic activity against MSC can be used as a biomarker to predict the response before MSC infusion, PGE2 levels in patient serum could be devised to monitor response after treatment.

The identification of such biomarkers will also harmonize the broad heterogeneity among MSC manufacturing processes across different centers (187). This will be a crucial pre-requisition for rigorous and scientific reproducibility across studies by which assess the difference between MSC preparations, MSC sources, administration regimen and doses.

So far, the translation of the in vitro models of MSC immunosuppression has failed to provide assays able to guide patient stratification. The discovery that MSC apoptosis is essential for MSC therapeutic efficacy in vivo represents a paradigm shift in the MSC field. This does not necessarily imply that it is the only possible mechanism and we cannot exclude the co-existence with other soluble-mediated mechanisms. However, this provides a reconciliation of the paradox between absence of engraftment and activity thus giving a strong mechanistic base of apparently contradictory experimental observations. Furthermore, in agreement with clinical data, it strengthens the notion that it is the patient with his/her inflammatory environment who plays a crucial role in the final response. Most importantly, this novel mechanism can be easily translated into reliable biomarkers. While the ability of the recipient to generate apoptotic MSCs appears to be a requirement for the therapeutic efficacy and could be used to stratify patients for MSC infusions before the treatment, the PGE2 levels in patient after MSC infusion could be exploited to monitor the response and provide a tool for detecting early treatment failures (Figure 2).

These new biomarkers may represent the dawn of a new era of MSC use in GvHD. However, we are only scratching the surface of the challenge in our attempt to improve the use of MSCs in GvHD and other inflammatory diseases. New questions need to be addressed and new paths identified to pave the way. Gaps are also yet to be filled regarding the relationship between MSC apoptosis and the classical “cytokine licensing.” A follow-on question regards the extent and the durability of the tolerogenic environment created by apoptotic MSCs. The restricted location of MSC apoptosis does not seem to reconcile with the systemic effects on inflammation. Answers to these questions will certainly provide novel insights and will lead us to the improvement of the available biomarkers or the discovery of new and more precise assays.

**AUTHOR CONTRIBUTIONS**

TC, GB, CG, FD, and AG designed the scope and context of this review. TC, GB, CG, and AG wrote the manuscript. TC, FD, and AG participated in manuscript revision and editing. MB, FD, and AG supervised the process. All authors contributed to the article and approved the submitted version.
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