Multipotent mesenchymal stromal cell therapy for steroid-refractory acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation

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Steroid-refractory acute graft-versus-host disease is a severe complication after allogeneic stem cell transplantation. So far, its treatment remains very challenging since the current therapies do not offer significant benefits. Among the most recent approaches, multipotent mesenchymal stromal cell-based therapy has attracted great interest over the past decade. Here, we briefly reviewed the current knowledges about the immunomodulatory properties of multipotent mesenchymal stromal cells as well as results of preclinical and clinical studies having assessed their efficacy to modulate steroid-refractory acute graft-versus-host disease.

(Belg J Hematol 2016;7(6):229-35)

Introduction

Allogeneic hematopoietic stem cell transplantation (alloHSCT) offers potential curative treatment for a wide range of haematological disorders. However, its success is limited by risks of post-transplant graft-versus-host disease (GVHD), a systemic syndrome in which donor's immune cells recognise and attack healthy tissues in the immunocompromised host.

Acute GVHD (aGVHD) mostly occurs during the first 100-180 days after alloHSCT and manifests as strong inflammatory lesions mainly of the skin, gut and liver. It can be clinically scored from grade I to IV, according to the severity of organ signs and dysfunctions. Clinically significant grade II-IV aGVHD is a major cause of transplant-related morbidity and mortality after alloHSCT. Standard first-line treatment for aGVHD consists of high dose systemic corticosteroids (1-2 mg/kg bodyweight). Unfortunately, steroids fail to produce sustained responses in approximately 30-50% of patients.[1] Although a number of immunosuppressive drugs have been tested as second-line therapy for treating steroid-refractory aGVHD (SR-aGVHD) (including antithymocyte globulin, infliximab, sirolimus/everolimus, cyclophosphamide, and extracorporeal photopheresis, among others), the final outcome for SR-aGVHD still remains

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Conflict of interest: The authors have nothing to disclose and indicate no potential conflict of interest.

Keywords: acute graft-versus-host disease, allogeneic stem cell transplantation, multipotent mesenchymal stromal cells.

Acknowledgments: Servais is Postdoctoral Researcher, Grégoire is Televie PhD student and Baron is Senior Research Associate at the National Fund for Scientific Research (FNRS) Belgium.

poor.[2] Therefore, there is a real need for new potent salvage approaches, and one that has attracted great interest in the last few years is multipotent mesenchymal stromal cell (MSC) therapy.

MSC biology

MSCs are non-hematopoietic multipotent progenitors that are characterised by the ability to differentiate into various cells and tissues, such as chondrogenic, osteogenic and adipogenic lineages. So far, no specific marker for defining MSCs has been described, although the International Society for Cellular Therapy (ISCT) has proposed minimal criteria that include:

1) plastic-adherence when maintained under standard culture conditions;
2) expression of CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules; and
3) ability to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro*.[3]

While bone marrow (BM)-derived MSCs are key players in the stem cell niches, MSCs can also be *ex vivo* expanded from virtually any connective tissue, such as umbilical cord, umbilical cord blood, placenta, adipose tissue and skin, among others.[4]

Over the last decades, MSCs have proven unique properties including hematopoietic support, regenerative potential as well as migration toward sites of inflammation. MSCs were initially used for tissue repair and regenerative medicine. However, one of the highlights of MSC functions was the increasing evidence that these cells could also mediate potent immune modulatory effects. This prompted their use in numerous immune-mediated conditions, including chronic inflammatory autoimmune diseases and GVHD after alloHSCT.

Immunomodulatory properties of MSCs

MSCs display a broad spectrum of immunomodulatory properties by interacting with various immune cells, belonging both to the adaptive and innate immune system (*Figure 1*). Here is a brief summary of some key properties, in an attempt to better understand the rationale of using MSC therapy for the treatment of aGVHD.

First studies demonstrated that MSCs were able to suppress T-cell proliferation and activation as well as to induce T-cell anergy and apoptosis.[5,6] Further, it was reported that MSCs could also regulate helper T (Th) cell differentiation, mainly by limiting type 1 and type 17 and favouring type 2 and regulatory polarisation. As an important subpopulation of Th cells, regulatory T cells (Tregs) play a crucial role in inducing peripheral immune tolerance. MSCs were reported to promote natural and inducible Treg generation. Finally, while first studies mainly focused on the effects of MSCs upon the peripheral T-cell compartment, recent works suggested an additional protective role of MSCs upon the central T-cell compartment, by supporting and improving thymic functions.

MSC also demonstrated modulatory effects upon B cell activation, proliferation and immunoglobulin production.[7] Recently, it was documented that MSCs could increase interleukin (IL)-10 producing CD5+ regulatory B cells (Bregs).[8]

Another mechanism of MSC immunomodulatory functions likely resides in their ability to interact with innate immune cells.[9] Studies have shown that MSCs could inhibit dendritic cell (DC) maturation from both CD34+ and monocytes precursors, decrease their ability to prime T cells (by impairing their migration and down-regulating their costimulatory molecule expression), and modify their secretion profile (induction of IL-10 secretion) to induce a tolerogenic DC phenotype. MSCs could also increase IL-10 secretion by macrophages and polarise them into anti-inflammatory M2 macrophages. Finally, MSC were reported to inhibit natural killer (NK) cell proliferation, cytotoxic functions and cytokine secretion.

Mechanisms by which MSCs regulate all of these cell subsets include both direct cell-to-cell contacts (such as through their expression of Fas, programmed death ligand 1 (PDL-1), vascular cell adhesion molecule-1 (VCAM-1) and galectin-1, among others) and paracrine effects by production of several soluble factors (such as indoleamine 2, 3-dioxygenase (IDO), nitric oxide (NO), tumour necrosis factor-stimulated gene 6 (TSG-6), transforming growth factor-β1 (TGF-β1), prostaglandin E2 (PGE2), IL-6, human leukocyte antigen (HLA)-G5, among others).[10] Recently, investigators have also characterised exosome-like microvesicles that are released by MSCs and contain bioactive molecules (such as cytokines, growth factors, messenger RNA, and microRNA, among others).

Besides, recent studies have demonstrated that MSCs displayed high variability and plasticity in their immuno-

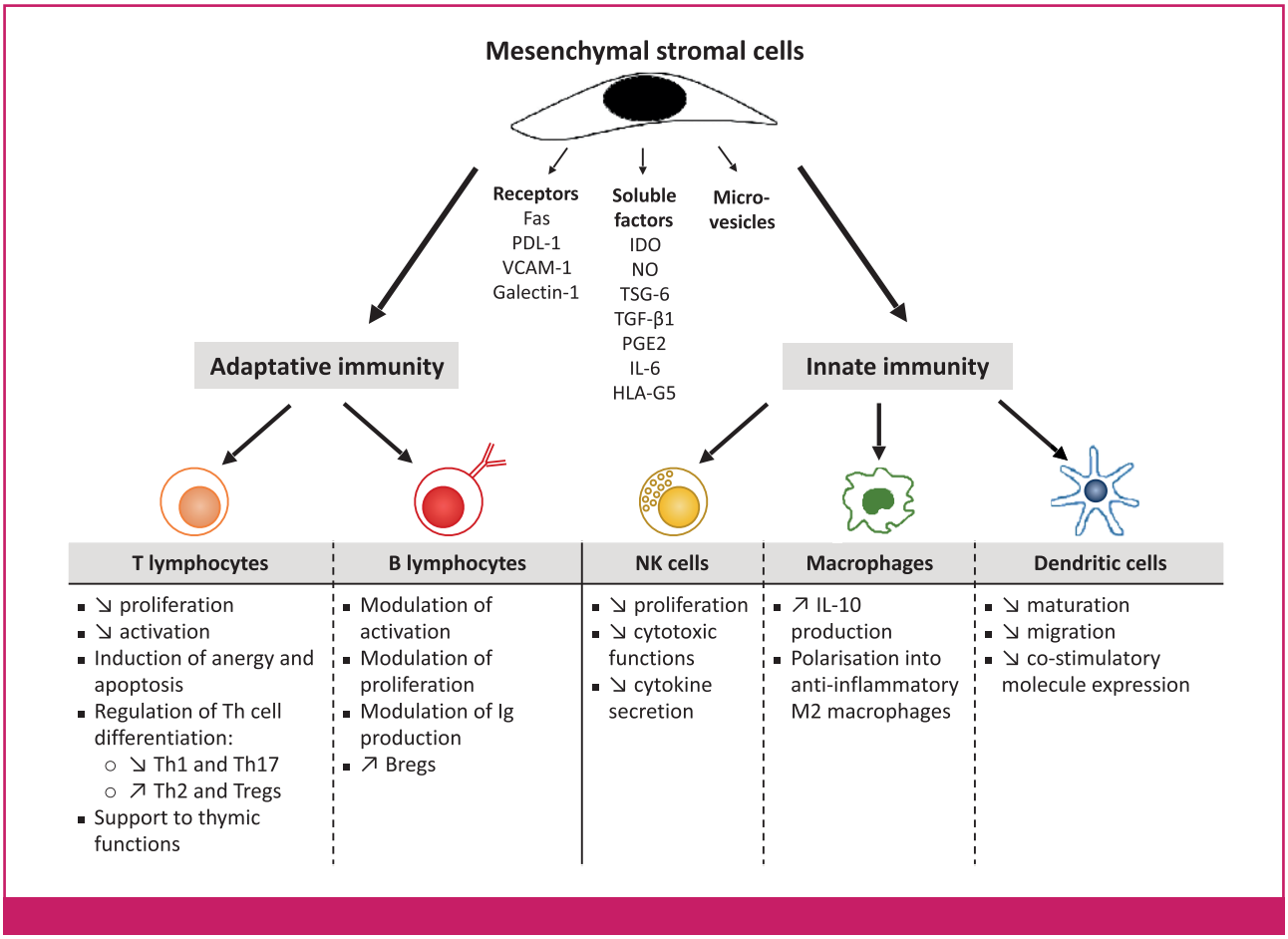

Figure 1. Immunomodulatory properties of multipotent mesenchymal stromal cells (MSCs).
Bregs: regulatory B cells ; IDO: indoleamine 2, 3-dioxygenase; Ig: immunoglobulins; IL: interleukin; HLA: human leukocyte antigen; NO: nitric oxide; PDL-1: programmed death ligand 1; PGE2: prostaglandin E2; TGF-β1: transforming growth factor-β1; Th: T helper; Tregs: regulatory T cells; TSG-6: tumour necrosis factor-stimulated gene 6; VCAM-1: vascular cell adhesion molecule-1.

modulatory effects, depending on several parameters such as their tissue origin, their culture conditions, and their environment. For example, adipose tissue-derived MSC were reported to have the most potent suppressive effects on T-cell proliferation and DC differentiation *in vitro*.[11] Short-term hypoxic pre-conditioning of MSCs seemed to enhance their migration and engraftment capacities *in vitro* and *in vivo*.[12] There is also increasing evidence that MSCs are sensors of inflammation and that they may modulate their functions, switching their phenotype to either an anti-inflammatory/immunosuppressive or a pro-inflammatory/immune-stimulating profile, depending on the environment.[13] Accordingly, different toll-like receptor agonists were identified to influence MSC functions. Inflammatory cytokines such as IFN-γ, TNF-α and IL-1β were reported to increase secretion of chemokine receptor ligands ICAM-1, CXCL-10, and CCL-8, as well as production of immunosuppressive IDO by MSCs. On the other hand, it was observed that, in the presence of IFN-γ, MSC could express major histocompatibility complex (MHC) class II molecules, act as antigen-presenting cells and stimulate T-cell proliferation. As described in pre-clinical GVHD model, a narrow window exists for MSC in which adequate levels of inflammatory IFN-γ can license them to acquire immunosuppressive effects.

MSC therapy for the treatment of aGVHD after alloHSCT

The pathophysiology of aGVHD is complex and involves a complex network of sensors (i.e. both donor's and recipient's DCs), mediators (i.e. helper T cells) and effectors (i.e. cytotoxic T cells, NK cells, macrophages, and granulocytes) of immune reactions, as well as tolerogenic actors (i.e. Tregs, Bregs) that mitigate the process. As described above, MSCs were reported to potentially act on each of these cell subsets. Besides their

immunomodulatory properties, their ability to home to sites of inflammation and injury and to stimulate tissue repair, have made them an attractive strategy to explore in the treatment of aGVHD. Moreover, MSCs are considered as immunoprivileged because they express low levels of human leukocyte antigen (HLA) class I molecules and do not express HLA class II antigens under normal circumstances. Hence, they can escape immune rejection and therefore can be transferred across HLA barriers, which is mandatory for 'off-the-shelf' cellular therapy.

Pre-clinical studies

A number of pre-clinical studies using various mouse models have assessed the efficacy of MSCs to mitigate aGVHD. Results were variable, with some studies having reported benefits while others not.[14,15] However, murine MSCs differ from human MSCs in several instances, including lower *in vitro* immunosuppressive activity, higher tendency to undergo immortalisation and transformation and absence of expression of IDO. A recent study demonstrated that there is a phylogenetic distinction of IDO and inducible nitric oxide synthase (iNOS) function in MSC-mediated immunosuppression in mammalian species: MSCs from monkey, pig, and human employ IDO to suppress immune responses, whereas MSCs from mouse, rat, rabbit, and hamster utilise iNOS.[16] This has to be taken into account when choosing appropriate animal models for preclinical studies of MSCs. Studying human MSCs in humanised mouse models of xenogeneic GVHD (xGVHD) may offer the opportunity to circumvent these issues. Nevertheless, conflicting results were also observed with MSC therapy in these models.[17,18] Various factors, including the experimental model, source of MSC (BM, umbilical cord or cord blood), MSC dose, timing of infusion, MSC manufacturing, number of infusions (single or repeated) and the resting or activated status of MSCs might have contributed to result heterogeneity among these studies. Taken together, most of these studies in mice tended to suggest that the co-injection of resting (non-activated) MSCs with the transplant failed to mitigate GVHD, while co-injection of pre-activated (i.e. with IFN-γ) MSCs, and repeated MSC injections at the time of and after transplantation showed clinical benefit in some but not all studies.[14,15,17,18] However, repeated MSC injections failed to prevent lethal GVHD in a pre-clinical canine model of dog leukocyte antigen-haploidentical transplantation.[19]

Clinical experience in the setting of SR-aGVHD

Since the pioneer publication by Le Blanc *et al*. having reported the success of infusions of HLA-haploidentical MSCs to rescue a paediatric patient with grade IV aGVHD refractory to multiple lines of treatment, a number of phase I-II studies have been performed to assess the efficacy of allogeneic MSCs as SR-aGVHD salvage therapy.[20-23] Among those, one of the largest multicentre studies (55 patients) was conducted by the European Group for Blood and Marrow Transplantation (EBMT) Consortium and reported an overall response rate (ORR) of aGVHD of 71%.[24] MSC were collected from either HLA-identical siblings, HLA-haploidentical relative, or third-party HLA-mismatched donors. The median dose of MSC per infusion was 1.4 (range 0.4-9) x 10^6 MSCs/kg recipient's weight and patients received either single or multiple (up to five) infusions (about half and half of the cohort, respectively). Besides this trial, several additional studies have reported similar encouraging results, while some others have failed to demonstrate benefits with MSC therapy.[21-23] Among the negative trials, a recent report of the German group did not show a difference in ORR of SR-aGVHD with MSC therapy from third-party donors when compared to the historical cohort without MSC treatment.[25] In Belgium, we recently evaluated MSC therapy (one or two infusions of 1-4 x 10^6 MSCs/kg) for treating SR-aGVHD in a phase II study, conducted on behalf of the Belgian Hematological Society. Thirty-three patients were included from seven Belgian and one Dutch centre, between February 2008 and November 2014. The study failed to meet its primary clinical endpoint of achieving a rate of sustained response (lasting for at least one month) of at least 40%. The ORR of SR-aGVHD to MSC therapy was 46.9% but the rate of sustained response was only 20.7% (unpublished data). Accordingly, the results from the sole phase III trial actually completed were also somewhat disappointing. This randomised (2:1), placebo-controlled, multicentre US phase III trial evaluated the potential of industrial MSCs (Prochymal®) in addition to institutionally selected second-line treatment to treat SR-aGVHD in 244 patients (ClinicalTrials.gov: NCT00366145). Patients either received eight infusions of placebo or 2×10^6 MSC/kg over four weeks, with additional four weekly infusions after day 28 in cases of partial responses. Although the study was completed in 2009, results have only been published in abstract form so far.[26] Unexpectedly, the study did not observe a significant difference in the rate of overall complete

and durable (≥28 days) responses between the two groups [primary endpoint; 35% in the MSC versus 30% in the placebo groups (P=0.3)]. Subgroup analyses suggested higher response rate to MSCs among patients with liver and/or gastrointestinal aGVHD. The reported failure to reach the primary endpoint in this study has led to significant scrutiny of the design of this clinical trial and of the source of MSCs (cryopreserved, highly passaged industrial manufactured MSCs from a small number of donors) and question the real effects of MSCs in aGVHD.[27]

Taken together, it still remains difficult to have a precise opinion about the efficacy of MSC therapy in SR-aGVHD. Several recent publications have tried to summarise results from published clinical trials.[21-23] Of the thirteen studies they compiled in their meta-analysis, Chen *et al*. reported that 205 of the 301 included patients exhibited overall response (136 complete and 69 partial responses) of SR-aGVHD to MSC therapy, suggesting that MSC therapy might be an acceptable treatment for SR-aGVHD.[21] However, caution is warranted as there may be a trend toward selective publication of positive trials in this field. Other large randomised controlled trials are ongoing and should better define the impact of this treatment modality in SR-aGVHD.

On the other hand, the marked heterogeneity between studies also has to be highlighted, such as the absence of consistency in MSC manufacturing (source, industrial or academic production, culture media, number of passages, cryopreservation), in MSC infusional protocols (dosages, infusional schedules with single or repeated infusions), in criteria for defining aGVHD response and in the management of concomitant immunesuppressive therapy.[23] This heterogeneity might have participated in discrepancies between results observed from clinical trials. Standardisation in MSC production and administration protocols may be relevant for future studies, as well as new reliable tests for predicting their immunosuppressive potency before their infusion.

Potential factors impacting MSC efficacy against aGVHD

An array of factors is thought to influence MSC effects and might have contributed to discrepancies between clinical studies assessing MSC therapy in SR-aGVHD. First, investigators were interested in identifying patient characteristics that might predict response to MSC therapy. In recent meta-analyses, MSC therapy was shown to have better efficacy in patients with lower grade aGVHD and only skin involvement, as well as in paediatric as compared with adult patients.[21,22]

Several observations have suggested that MSC manufacturing and processing might also impact their nature and their functions. Some reports have shown that adipose tissue-derived MSCs had better immunosuppressive capacities than BM-derived MSCs *in vitro*.[11] The efficacy of adipose tissue-derived MSCs for controlling clinical aGVHD has only been assessed in small clinical studies, but results were encouraging.[28] Whether they could be more efficient against aGVHD than BM- or cord blood-derived MSCs in the clinical setting is not known and remains to be explored. Culture conditions, such as oxygen tension, temperature, and medium composition have also been explored. Specifically, the use of foetal bovine serum (FBS) or other media containing xenoantigens has brought some concerns about risks of increased immunogenicity and lower efficacy of MSCs. The use of human blood-derived supplements, such as platelet lysate, has been explored as an alternative but did not seem to result in better clinical response in the setting of SR-aGVHD.[21,22] Recently, the issue of passage number of MSCs has also been raised, and there were suggestions that use of early passaged MSCs might have greater efficacy against aGVHD.[29] With continuous expansion pressure, it is conceivable that senescence and epigenetic reprogramming occurred that might lead to cell replicative exhaustion and to loss of therapeutic efficacy. Induction of a senescence-associated pro-inflammatory secretory phenotype is also plausible. Hence, the scale of product expansion can be one parameter that has contributed to discrepancies in results between the phase III US trial having used highly expanded industrial manufactured MSCs (Prochymal) and most of the phase II studies having used early-passaged MSCs manufactured by academic centers.[27] Moreover, although clinical trials almost universally used cryopreserved MSCs, freezing and thawing procedures were also reported to potentially affect MSC viability and immunosuppressive properties.[30] Eventually, dosage and infusional schedule of MSC administrations have also been explored as parameters influencing MSC efficacy in clinical studies but no significant correlation was observed between response of aGVHD and dose or the number of MSC infusions in meta-analyses.[21,22]

Researchers have also been interested in identifying donor's characteristics that might predict response to

MSC therapy. Most clinical studies did not report influence of donor sex or age. However, age likely impacted the population doubling level of MSC *in vivo* in the BM, before collection. This is why younger donors are usually preferred, in a way to collect MSCs that have undergone only limited rounds of division *in vivo*. As mentioned above, it has been suggested that licensing of human MSCs with IFNγ markedly potentiates their immune suppressive properties by inducing IDO expression. A recent study showed that the magnitude of IDO responsiveness arising from IFNγ activation was not uniform among MSC products from different donors.[31] Hence, MSCs derived from low IDO inducers may be substantially less potent for controlling aGVHD than cells derived from high inducers. Therefore, IFNγ responsiveness might be an interesting parameter to consider when choosing a potential MSC donor, but this has to be confirmed in clinical studies. Recently, the transcription factor Twist1 was identified as a key regulator of MSC properties, with high Twist1 expression associated with proliferative and pro-angiogenic capacities and low Twist1 expression with anti-inflammatory and immunomodulatory activities.[32] Interestingly, Twist1 expression was decreased by IFN-γ pre-treatment of MSCs. These observations led the authors to develop a Clinical Indications Prediction (CLIP) scale based on Twist1 expression levels to predict how donor-to-donor heterogeneity could impact the therapeutic efficacy of MSC populations. Hence, MSCs expressing low levels of Twist1 might be the most effective for controlling aGVHD. This hypothesis has to be confirmed in further studies. Taken together, this is why MSC production and banking from multiple donors (instead of from a small number of donors) is usually preferred by most academic MSC banks, in a way to guarantee variability of donors and to avoid potency bias if only one donor is used to produce a multiplicity of MSC doses.[30]

Eventually, while MSC origin, manufacturing and delivery likely impact their clinical efficacy, there is increasing evidence that attention should also be payed to MSC immune modulation in varying inflammatory environments. Inflammatory conditions may change between aGVHD patients (i.e. depending on the extent of the lesions) and throughout the course of aGVHD pathogenesis. Hence, monitoring individual immune and inflammation microenvironment may be an interesting approach for the future, to predict MSC efficacy *in vivo* and to adjust the best timing of MSC administration.[33]

In addition, most of the immunosuppressive drugs (including glucocorticosteroids) used for controlling aGVHD exert potent anti-inflammatory effects. Therefore, one may question the real impact of concomitant administration of such immunosuppressive agents with MSC therapy on the efficacy of this cellular approach *in vivo*.

Conclusion

SR-aGVHD after alloHSCT is associated with very dismal outcomes. So far, its treatment continues to be very challenging and there is no standard second-line therapy. Over the last two decades, MSC infusions have been considered a promising therapy for SR-aGVHD since several pre-clinical and clinical phase I/II studies have reported encouraging data. However, such positive results were not consensually observed in all studies and therefore controversy still remains. Heterogeneity in MSC manufacturing and administration protocols as well as in donor and recipient characteristics might have contributed to results ambiguity. Many questions remain to be answered in the performance of MSCs for SR-aGVHD, including optimal donor selection, culture conditions, timing for application, dose, route of delivery as well as the impact of freezing/thawing process and of concomitant administration of other immunosuppressive drugs. Adaptation and standardisation of these parameters may be relevant for further studies. Additionally, the recent understanding that MSCs are highly responsive to inflammatory environmental stimuli provides a new paradigm for MSC-based cellular therapy and challenges MSC clinical applications.

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Key messages for clinical practice

1. SR-aGVHD after alloHSCT is associated with very dismal outcomes.
2. So far, there is no standard therapy for SR-aGVHD.
3. MSC have demonstrated potent immunosuppressive effects *in vitro* as well as in several pre-clinical and phase I-II clinical studies.
4. Further large-scale randomised prospective studies are needed to validate the use of MSC in SR-aGVHD.
5. A better understanding of MSC biology is also warranted to define optimal MSC production and administration protocols.

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