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REVIEW

Mesenchymal stem cells for the treatment of systemic lupus erythematosus: is the cure for connective tissue diseases within connective tissue?

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Abstract

Mesenchymal stem cells (MSCs) are now known to display not only adult stem cell multipotency but also robust anti-inflammatory and regenerative properties. After widespread *in vitro* and *in vivo* preclinical testing in several autoimmune disease models, allogenic MSCs have been successfully applied in patients with severe treatment-refractory systemic lupus erythematosus. The impressive results of these uncontrolled phase I and II trials - mostly in patients with non-responding renal disease - point to the need to perform controlled multicentric trials. In addition, they suggest that there is much to be learned from the basic and clinical science of MSCs in order to reap the full potential of these multifaceted progenitor cells in the treatment of autoimmune diseases.

Introduction

Patients with systemic lupus erythematosus (SLE) remain at increased risk for premature death [1], particularly among young adults [2]. Up to 45% of cases present early end organ damage, related to persistent activity or kidney disease [3]. Renal involvement, which occurs in 40 to 50% of patients in most series, is associated with mortality rates approximately eight times higher than expected [2], and remains only partially responsive to the best available treatments. Indeed, therapy with cyclophosphamide has not improved patient survival compared with cortico-steroids [4], and the largest controlled trial comparing

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mycophenolate mofetil with intravenous cyclophospha-mide in lupus nephritis achieved complete remission in only 8.6% and 8.1% of patients, respectively [5]. Undoubtedly there is a need for safer and more effective treatments for SLE.

Mesenchymal stem cells

Mesenchymal stromal cells, originally described in the 1960s as bone forming cells in the bone marrow [6], are now called multipotent mesenchymal stromal cells, or more commonly mesenchymal stem cells (MSCs) since they display adult stem cell multipotency. Thus, they differentiate into bone, cartilage and other connective tissues [7]. Unlike hematopoietic stem cells, which origi-nate from bone marrow, MSCs can also be isolated from a variety of other tissues, such as umbilical cord or adipose tissue, and can be extensively expanded *in vitro* by up to 50 cell doublings without differentiation [8]. While these properties initially put MSCs center stage of an alleged era of regenerative medicine, the unexpected findings of Bartholomew and colleagues in 2002 [9] pointed to new features of these progenitor cells, the consequences of which are still being revealed in several areas of medicine. MSCs were found to escape T-cell recognition, suppress T-cell response to mitogens and also to prolong skin graft survival in baboons. In spite of a wide array of immunomodulatory effects that were subsequently proven to affect T and B lymphocytes, natural killer and antigen-presenting cells [10,11], MSCs remain hypoimmunogenic since they express low levels of major histocompatibility (MHC) class I molecules and do not express MHC class II or co-stimulatory (CD40, CD40L, CD80 or CD86) molecules [12]. Since the effects on immunocompetent cells are not MHC restricted, allogenic MSCs are widely used with no need to match them with host human leukocyte antigens (HLAs). The mechanisms underlying these effects are a subject of great scientific interest, as reviewed elsewhere in this issue, but apparently involve both cell contact and soluble

factors, including indoleamine 2,3-dioxygenase, prosta-glandin E2, nitric oxide, transforming growth factor (TGF)-β1, IL-10, soluble HLA-G, and IL-1 receptor anta-gonists [13,14]. Also, several growth factors, such as hepatocyte growth factor, vascular endothelial growth factor (VEGF), insulin-like growth factor, epidermal growth factor, basic fibroblast growth factor and stromal cell-derived factor-1, among others, have been implicated in the modulatory and reparative effects of MSCs [15].

Recently, several studies have identified critical roles for microRNAs (miRNAs) involved in proliferation, migration and differentiation of MSCs, suggesting that they might play an important role in the acquisition of reparative MSC phenotypes [16].

Therapeutic use of MSCs in autoimmune and inflammatory diseases

Given their vast proliferative potential, extensive immuno-suppressive properties, and also the ease of access to proper tissue sources, therapies with autologous or allogenic MSCs have been tested in a variety of immune-mediated disease models, including experimental allergic encephalomyelitis [17,18] - a model of multiple sclerosis - diabetic NOD/SCID mice [19], collagen-induced arthritis [20,21], and several lupus murine models [22-28]. Results have been mainly encouraging, but not altogether consis-tent, particularly in the case of arthritis [29], and lupus mice [26,27].

At the time of writing this review, 141 registered human trials on MSCs were found at the National Insti-tutes of Health ClinicalTrials.gov website [30], including 13 for graft versus host disease (GVHD), 10 for diabetes, 7 for Crohn's disease or ulcerative colitis, 5 for multiple sclerosis, 2 for amyotrophic lateral sclerosis, one each for Sjögren syndrome and systemic sclerosis and two for SLE. Some of these trials point to non-immune-mediated conditions that are associated with tissue injury, such as hepatic cirrhosis, myocardial infarction or congestive heart failure. In several instances it has become apparent that MSCs are not necessarily replacing diseased tissues or differentiating into separate cell lineages, but seem to exert a complex pattern of trophic, regenerative and anti-inflammatory effects [31,32].

In humans, the most studied application for MSCs is GVHD, a complication of hematopoietic stem cell trans-plantation in which donor T cells attack an immuno-compromised and genetically disparate recipient [33]. In 2004, Le Blanc and colleagues [34] treated a 9-year-old boy with severe treatment-resistant acute GVHD of the gut and liver with third party haplo-identical mother-derived MSCs. Clinical response was striking, with improvement of liver and intestinal function. The most recent placebo controlled trials confirmed the significant improvement in liver and gastrointestinal GVHD, but did

not reach significance for durable complete responses or other primary endpoints [35].

Mesenchymal stem cells in systemic lupus erythematosus

Perhaps the most remarkable results for human MSC therapy are now emerging from the latest clinical trials in severe, treatment-refractory SLE [36,37]. While these are still small, uncontrolled and non-multicentric studies, the recent reports of successful MSC treatment in other inflammatory and scarring conditions that are typical of the SLE spectrum [38,39] lend support to these notoriously favorable outcomes. These trials also highlight the need to advance the clinical science of stem cell therapy and underscore the challenge of identifying specific mechanisms of action, given the multi-tiered effects of cellular therapies *in vivo* [40].

While in the past the connective tissue was assigned a low rank among organized tissues, nowadays it seems to harbor far reaching properties. Undoubtedly, when Dr Paul Klemperer suggested that the histopathological connective tissue changes found in SLE were common to the 'obscure maladies that collectively are called diseases of the connective tissue or collagen diseases' [41], little did he know that a cure for such diseases might also be found within connective tissues!

Animal models of disease

While the MSCs derived from SLE patients and diseased mice are still immunosupressive *in vitro* [42], they are abnormal in terms of phenotype, proliferation and differ-entiation [43-45]. Sun and colleagues have forwarded the hypothesis that an impaired bone marrow MSC niche contributes to disease development in human [43] and murine SLE [23]. They describe in Fas-deficient MLR/lpr mice a significant osteoporosis phenotype with osteoclast activity and T cell over-activation that does not respond to cyclophosphamide treatment, but is corrected by MSC transplant [23]. Even if this assumed MSC deficiency is only a consequence of immune activation in SLE, this rationale has supported the use of allo- or xenogeneic - instead of autologous - MSCs for the treatment of SLE [23]. For example, in MRL/lpr mice, allogenic mouse or human MSCs derived from bone marrow (BM-MSCs), umbilical cord (UC-MSCs) or exfoliated deciduous teeth have all been highly effective in reducing or even normalizing serum autoantibodies, proteinuria, renal pathology and survival of diseased animals [22-25]. In contrast, the NZB/W F1 strain, considered the murine model that most closely resembles human SLE, has shown diverging results. For example, human UC-MSCs delayed disease and alleviated lupus nephritis [27], while allogenic murine BM-MSCs (from C57BL/6J mice) did not affect proteinuria or double-stranded DNA (dsDNA)
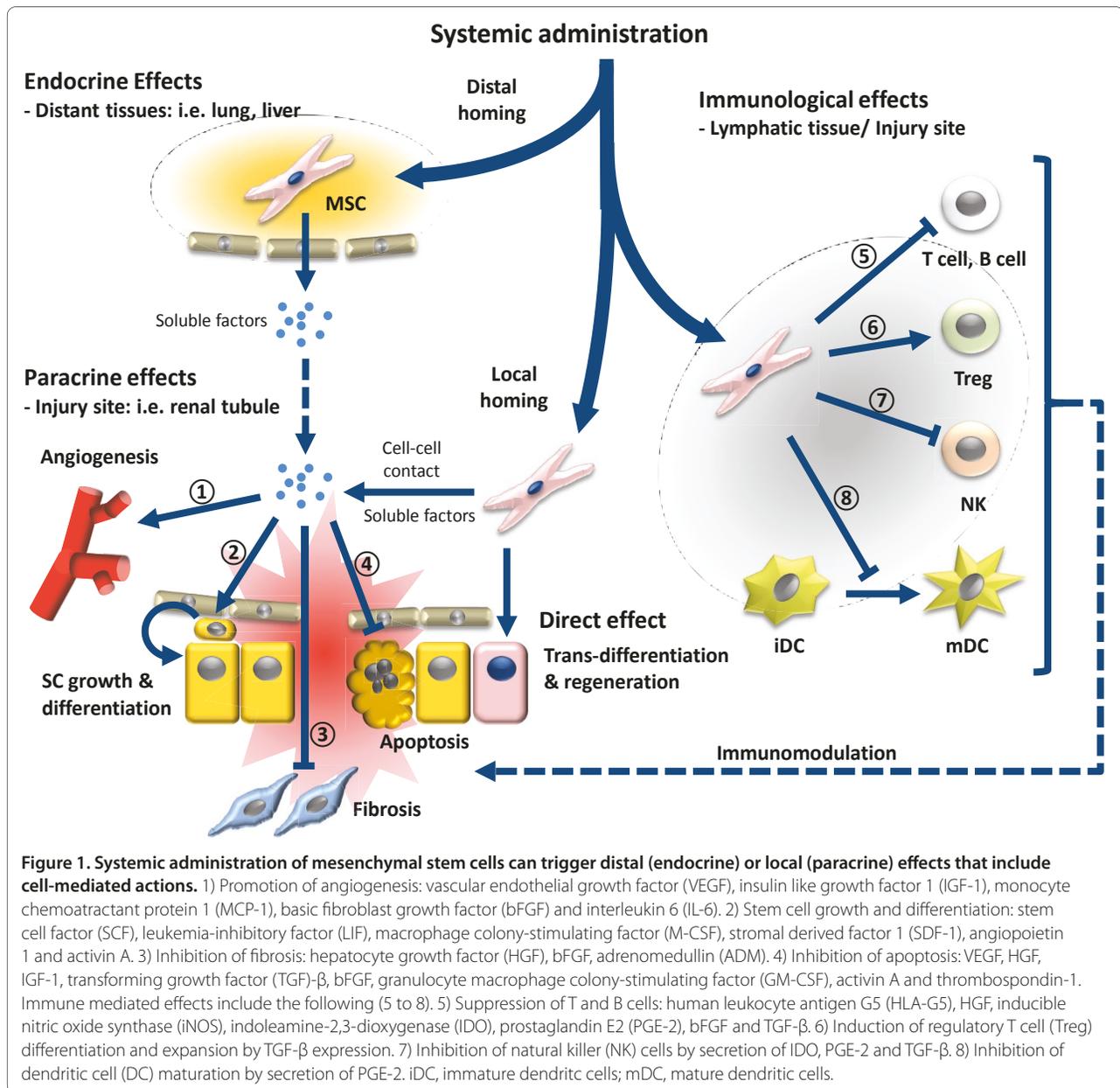
levels, but still improved renal function [28]. Surprisingly, BM-MSCs from another strain (BALB/c mice) had opposite effects, enhancing anti-dsDNA antibody production and worsening disease and kidney pathology [26].

Human systemic lupus erythematosus

Prompted by the positive results in the Fas-deficient MRL/lpr mice treated with human MSCs from healthy individuals [22], Sun and colleagues [23] treated four patients with active disease and lupus nephritis (24 hour urine protein ≥1 g and/or serum creatinine ≥1.5 mg/dl) that was unresponsive to monthly intravenous cyclophosphamide (0.75 g/m^2) and oral prednisone (≥20 mg/day) for 6 months. All patients received one infusion of ≥1 × 10^6 BM-MSCs (from healthy family members) per kilogram of body weight. The Systemic Lupus Disease Activity Index (SLEDAI) at 1, 6 and 12 months follow-up improved significantly, as did urinary protein, and also CD4+ Foxp3+ T regulatory (Treg) cell counts at 3 months follow-up. Prednisone and cyclophosphamide were reduced, and the latter even suspended in two patients. None had complications after 12 to 18 months follow-up. These encouraging results led to a larger phase I open trial in 15 patients - including the first 4 cases reported - with refractory disease as described above, except that one-third of patients had also failed oral mycophenolate mophetil (1 to 2 g/day for 3 months) [37]. All cases fulfilled the previously stated criteria for refractory renal disease except one with only refractory thrombocytopenia (24 × 10^9/L). Non-renal manifestations included arthritis, severe skin disease, serositis and ten cases with non-responsive cytopenias. Patients received one intravenous infusion of 1 × 10^6 allogeneic BM-MSCs per kilogram body weight (harvested from passages 3 to 5) from non-HLA-matched healthy family members. Subsequently, steroids were reduced to 5 to 10 mg/day maintaining lower dose cyclophosphamide (0.4 to 0.6 g) for 2 to 3 months. Mean follow-up reached 17.2 (3 to 36) months with no adverse effects, deaths or ensuing GVHD. Clinical and serological changes were quite dramatic for this group of patients with severe disease as gauged by an average baseline SLEDAI of 12.1 ± 3.3, in spite of daily prednisone (23 ± 5 mg) and immunosuppressive drugs. In 12 patients SLEDAI again improved significantly, to 3.2 ± 2.8 at 12 months ($P < 0.05$), remaining under 8 in all patients and even zero in four patients. Only one subject was able to discontinue immunosuppressants, remaining with inactive disease at 12 months on 5 mg daily prednisone. Two patients flared at 6 and 12 months, respectively. Quite surprisingly, 24 hour proteinuria (2,538.0 ± 382.3 mg at baseline) decreased significantly (1,430.7 ± 306.3; $P < 0.01$, n = 12) as soon as one week after MSC therapy - even preceding changes in anti-dsDNA antibodies - and continued to improve thereafter

until month 12. Glomerular filtration rate improved in two patients who had reduced values at study entry, as did creatinine levels in four subjects. Anti-dsDNA antibodies decreased significantly at 1 month ($P < 0.05$) and 3 months ($P < 0.05$) post-transplant. Treg cells, which have been found to be quantitatively and qualitatively deficient in active SLE [46,47], were restored at week 1 (from 2.56 ± 0.37 to 4.58 ± 0.51; $P < 0.05$, n = 13) as judged by the percentage of CD4+ Foxp3+ cells among peripheral blood mononuclear cells.

A second open trial from this group in Nanjing, China followed, reporting the use of UC-MSCs in severe lupus [36]. UC-MSCs are easily accessible, have high proliferative potential [48] and have been used with success in lupus mice [24]. Patients (n = 16) and entry criteria were similar to the previous study, though this time 5 of 15 renal cases had histological confirmation of proliferative nephritis, and 11 were preconditioned with cyclophosphamide (0.8 to 1.8 g intravenously) prior to MSC infusion. Subsequently, prednisone was reduced to 5 to 10 mg every 2 weeks and patients were kept on maintenance cyclophosphamide (0.6 to 0.8 g), which was able to be eventually discontinued in only three individuals. Mean follow-up was only 8.25 months. Significant improvement at 1 and/or 3 months was verified by SLEDAI score (two patients completed 2 years with scores <4), serum albumin, 24 hour urinary protein, serum creatinine (six patients), serum C3 (five patients) and anti-dsDNA antibodies. Baseline CD4+ Foxp3+ cells (Treg cells) increased significantly at 3 and 6 months, and a fall in serum IL-4 (with a non-significant increase of IFN-γ) was interpreted by the authors as indicative of improvement of pathogenic Th2 imbalance, though animal lupus models have shown the opposite cytokine change [27]. Finally, a case report from the group in Nanjing, but not from the realm of renal disease, draws further attention to the potency of MSC treatment: a 19-year-old girl with a recent diagnosis of SLE presented with massive diffuse alveolar hemorrhage unresponsive to methylprednisolone (160 mg/d for 4 days, 500 mg/d for 3 days) and intravenous immunoglobulin (20 g/day for 5 days) [49]. Repeated high-resolution chest computed tomography spanning 9 weeks showed diffuse bilateral alveolar infiltrates. After only one day of UC-MSC infusion (2 × 10^6/kg body weight) the patient's level of oxygen saturation rose from 71 to 91%, and 5 days later mechanical respiratory support was removed. Nine days later high-resolution chest computed tomography showed complete resolution. Recurrent pulmonary disease 6 weeks after being discharged - while on prednisone, cyclophosphamide and cyclosporin A - again responded promptly to MSC retreatment. This dramatic case underscores the need to unravel the biological components underlying the clinical effects of MSCs.

Figure 1. Systemic administration of mesenchymal stem cells can trigger distal (endocrine) or local (paracrine) effects that include cell-mediated actions. 1) Promotion of angiogenesis: vascular endothelial growth factor (VEGF), insulin like growth factor 1 (IGF-1), monocyte chemoatractant protein 1 (MCP-1), basic fibroblast growth factor (bFGF) and interleukin 6 (IL-6). 2) Stem cell growth and differentiation: stem cell factor (SCF), leukemia-inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF), stromal derived factor 1 (SDF-1), angiopoietin 1 and activin A. 3) Inhibition of fibrosis: hepatocyte growth factor (HGF), bFGF, adrenomedullin (ADM). 4) Inhibition of apoptosis: VEGF, HGF, IGF-1, transforming growth factor (TGF)-β, bFGF, granulocyte macrophage colony-stimulating factor (GM-CSF), activin A and thrombospondin-1. Immune mediated effects include the following (5 to 8). 5) Suppression of T and B cells: human leukocyte antigen G5 (HLA-G5), HGF, inducible nitric oxide synthase (iNOS), indoleamine-2,3-dioxygenase (IDO), prostaglandin E2 (PGE-2), bFGF and TGF-β. 6) Induction of regulatory T cell (Treg) differentiation and expansion by TGF-β expression. 7) Inhibition of natural killer (NK) cells by secretion of IDO, PGE-2 and TGF-β. 8) Inhibition of dendritic cell (DC) maturation by secretion of PGE-2. iDC, immature dendritic cells; mDC, mature dendritic cells.

Mechanisms of the therapeutic effects of MSC treatment

Despite the *in vitro* and *in vivo* evidence for a therapeutic effect of MSCs in SLE, the mechanisms by which MSCs exert their immunomodulatory and reparative effects are still incompletely understood, but most likely involve multiple mechanisms (Figure 1).

Proinflammatory 'licensing' of MSCs

In contrast to therapies that cause global immune suppression, MSCs have been dubbed 'smart' immune modulators since their suppressive effects require a previous 'licensing' step that occurs in the presence of an inflammatory environment, and is mediated by the secretion of specific cytokines [50]. Thus, IFN-γ, alone or together with tumor necrosis factor-α, IL-1α or IL-1β, are required to provoke the expression by MSCs of high levels of soluble factors involved in immunosuppression, such as indoleamine 2,3-dioxygenase, hepatocyte growth factor, TGF-β1 and nitric oxide [51-54]. The need for this activation step has been confirmed in a model of GVHD since recipients of IFN-γ$^{-/-}$ T cells did not respond to MSC treatment evolving into fatal GVHD [55].

Tipping of the Th1/Th2 balance

Although still controversial, an imbalance in IFN-γ and IL-4 cytokine levels, suggestive of a pathogenic T helper 2 (Th2) response, has been reported in SLE. Accordingly,

experimental data suggest that MSC therapy might ameliorate SLE by promoting the conversion from a Th2 humoral response to a Th1 cellular immune response through modulation of IL-4 and IFN-γ levels in effector T cells. Zhou and colleagues [22] showed that intraperitoneal infusion of human BM-MSCs in MRL/lpr mice decreased the production of IL-4 and increased IFN-γ in peripheral blood T cells. Sun and colleagues [36] reported similar findings with UC-MSC transplantation in patients with refractory SLE 3 months after treatment, also suggesting a polarization toward a Th1 phenotype that was associated with clinical improvement. However, Aggarwal and Pittenger [51] showed the opposite effect with the addition of human MSCs to differentiated effector T cells *in vitro,* and Chang and colleagues [27] found that UC-MSC transplantation in NZB/W F_1 mice was associated with an increase in the Th2 phenotype in the face of improving disease. The divergent results from these studies underline the complexity of both MSC-mediated effects and the immunopathogenesis of SLE.

Effects on CD4+ T cell populations: upregulation of Treg/Th17 ratio

Several studies have provided evidence of a quantitative and/or qualitative defect of Treg cells in human SLE, as well as an increased production of Th17 proinflammatory cells [46-47,56]. On the other hand, MSCs have been shown to induce the generation of functional Treg cells both *in vitro* and *in vivo* [21,57,58]. In MLR/lpr mice, the transplantation of MSCs from many sources (bone marrow, umbilical cord or exfoliated deciduous teeth), can restore Treg cells and induce a significant reduction in Th17 levels, consequently up-regulating the ratio of Treg/Th17 cells [23-25]. In human SLE, the transplantation of either allogeneic or autologous MSCs derived from bone marrow or umbilical cord also increases Treg cells, suggesting that this may be one of the mechanisms of the MSC-mediated improvement of disease [23,36,37]. However, in two patients with active but not highly inflammatory SLE, we reported that the infusion of autologous MSCs induced no amelioration in spite of generating a marked increase in Treg cells [59].

Mesenchymal stem cell homing and differentiation

Long-term persistence of autologous or allogenic MSCs after a single intravenous infusion has been described in baboons, with levels of tissue engraftment ranging from 0.1 to 2.7% [60]. However, in a chronic kidney disease model, only repeated injections were associated with functional improvement and cortical entrapment of MSCs at 5 weeks [39]. In NZB/W F1 lupus mice treated with 1×10^6 human UC-MSCs via the tail vein, Chang and colleagues [27] could evidence MSCs in kidney tissues at week 2 of infusion, but no long-term engraftment.

Even if MSCs protect and improve recovery from several models of acute and chronic renal injury [61,62], paracrine and endocrine effects seem most important, since conditioned medium from MSCs has been able to mimic the beneficial effects of stem cell therapy [63]. The intricacy of such endocrine factors *in vivo* has been elegantly illustrated by Lee and colleagues [64] in a mouse model in which the reduced size of myocardial infarction in response to the infusion of human MSCs was due to the secretion of the anti-inflammatory protein TSG-6 triggered by MSC entrapment in the lung.

Gene expression and growth factors

A number of genes and growth factors responsible for renal regeneration also seem to be involved in renal repair after the administration of MSCs [38]. High levels of angiogenic factors, such as VEGF, have been related to glomerulonephritis in SLE [65,66], and Zhou and colleagues [22] showed that transplantation of human BM-MSCs in MRL/lpr mice reduced the expression of VEGF and TGF-β and also deposits of fibronectin in glomeruli. In an ischemic model of chronic kidney disease we have shown that a single intravenous infusion of autologous MSCs triggers a significant increase in a group of nephrogenic proteins and transcription factors related to endothelial (VEGF and the angiopoietin-1 receptor Tie-2) and epithelial (bone morphogenetic protein-7, Pax-2, and basic fibroblast growth factor) differentiation, in association with a marked improvement of renal function [67].

Additionally, the importance of epigenetic regulatory factors in the control of biological processes and of the immune response has also been stressed. Common miRNA patterns of expression have been found in three different murine models of SLE [68], suggesting these might be targeted therapeutically. Since MSCs have been shown to secrete microparticles enriched in miRNAs [69], several authors have suggested that microvesicle-mediated transfer of mRNA from MSCs to target tissues might also participate in some of the processes involved in immunoregulation or in the recovery from kidney injury in response to stem cell treatment [70].

Conclusion

The results of the first clinical trials with MSC therapy in severe SLE are undoubtedly encouraging. However, the heterogeneity of MSCs as defined today and the intricate circuitry of cellular and humoral factors that mediate their presently known effects still point to many issues to be resolved in order to pave the way for cell therapy in SLE. Long-term safety concerns remain an issue, given the description of *in vitro* malignant MSC transformation [71] and the unknown interaction of regular immuno-supressants with single or repeated MSC therapy [72].

Along with the need for larger randomized controlled clinical trials, future advances from stem cell science can be expected to pinpoint significant MSC subpopulations and/or stem cell markers for regenerative or immuno-regulatory properties, as well as specific mechanisms of action [73]. Thus, assays for *in vitro* or *in vivo* MSC potency could be developed, leading the way to the use of more potent stimulated or primed pre-treated MSCs.

Abbreviations
BM-MSC, bone marrow-derived mesenchymal stem cell; dsDNA, double-stranded DNA; GVHD, graft versus host disease; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; miRNA, microRNA; MSC, mesenchymal stem cell; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Disease Activity Index; TGF, transforming growth factor; Th, T helper; Treg, T regulatory; UC-MSC, umbilical cord-derived mesenchymal stem cell; VEGF, vascular endothelial growth factor.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Both authors contributed to the writing of the manuscript and read and approved the final version.

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