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PAPER

Transplantation of umbilical cord mesenchymal stem cells alleviates lupus nephritis in MRL/lpr mice

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Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease, which, despite the advances in immunosuppressive medical therapies, remains potentially fatal in some patients, especially in treatment-refractory patients. This study found that transplantation of umbilical cord mesenchymal stem cells (UC-MSCs) has the same therapeutic effect as transplantation of bone marrow mesenchymal stem cells (BM-MSCs), which has been reported to be efficient in treating SLE-related symptoms in MRL/lpr mice. Multi-treatment (at the 18th, 19th, and 20th weeks of age) of 1×10^6 UC-MSCs was able to decrease the levels of 24-h proteinuria, serum creatinine, and anti-double-stranded DNA (dsDNA) antibody, and the extent of renal injury such as crescent formation in MRL/lpr mice. A lower, but still significant, reduction in these parameters was also observed in mice receiving a single dose of UC-MSCs (at the 18th week). UC-MSCs treatment also inhibited expression of monocyte chemotactic protein-1 (MCP-1) and high-mobility group box 1 (HMGB-1) expression in a similar fashion. UC-MSCs labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) were found in the lungs and kidneys 1 week post infusion. In addition, after 11 weeks post UC-MSCs infusion, human cells were found in kidney of UC-MSCs-treated mice. These findings indicated that UC-MSCs transplantation might be a potentially promising approach in the treatment of lupus nephritis, possibly by inhibiting MCP-1 and HMGB-1 production. *Lupus* (2010) **19,** 1502–1514.

Key words: high-mobility group box 1; lupus nephritis; monocyte chemotactic protein-1; regulatory T cell; systemic lupus erythematosus; umbilical cord mesenchymal stem cell

Introduction

Systemic lupus erythematosus (SLE) is a common and potentially fatal autoimmune disease characterized by multi-organ injuries including renal, cardiovascular, neural, musculoskeletal, and cutaneous involvements. Recent studies have demonstrated that overproduction of monocyte chemoattractant protein-1 (MCP-1) plays an important role in the pathogenesis of lupus nephritis (LN) in humans and animal models.[1–4] High-mobility group box chromosomal protein 1 (HMGB-1), actively secreted by macrophage/monocytes under inflammatory stimuli,[5] was recently found to act as a proinflammatory cytokine in SLE. The presence of anti-HMGB1 antibodies correlates with disease activity in SLE patients.[6] The induction of these two proinflammatory cytokines may play a pathogenic role in the development of nephritis in MRL/lpr mice. The pathology of SLE involves the destruction of targeted organ tissues and accumulation of autoreactive lymphocytes and immune complexes.[7,8] Regulatory T cells (Treg) play an essential role in maintaining immune homeostasis and preventing autoimmunity.[9,10] Defects in Treg development, maintenance or function have been associated

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with several human autoimmune diseases, including SLE.[11] Despite improved supportive care, aggressive immunosuppressive medical therapies, and new therapeutic interventions, a subset of SLE patients continue to suffer significant morbidity and mortality from active disease. Therefore, it is urgent to develop more effective therapy for SLE, especially for those who are refractory to treatment.

Mesenchymal stem cells (MSCs) are multipotent stem cells which are able to differentiate into a variety of cell types, including osteoblasts, chondrocytes, adipocytes, and myoblasts.[12–14] These cells have been shown to have immunosuppressive properties and to reduce inflammation.[15–18] Human MSCs suppress lymphocyte alloreactivity in vitro in mixed lymphocyte cultures through human leukocyte antigen-independent mechanisms.[15] Previous studies showed that MSCs could inhibit lymphocyte proliferation induced by a variety of mitogens.[18–20] Transplantation of ex vivo-expanded bone marrow MSCs (BM-MSCs) proved effective in treating acute graft-versus-host-disease (GVHD) by inhibiting T-lymphocyte function.[21–23] MSCs, which can produce important growth factors and cytokines, have a strong propensity to ameliorate tissue damage in response to injury and disease.[24] Relevant to this investigation, Kunter *et al*. demonstrated that intra-arterial infusion of adult MSCs provided a tool to accelerate glomerular healing in a rat model of mesangioproliferative glomerulonephritis.[25] BM-MSCs from SLE patients showed deficiency in terms of proliferation and differentiation. Normal BM-MSCs can be cultured for 40 passages without losing their productive motility. In contrast, BM-MSCs from SLE patients can only be cultured for about 10 passages, after which the culture shows senescence behaviour.[26] Our previous study has shown that the cytoskeleton and ultrastructure of SLE BM-MSCs are abnormal. Based on these findings, we hypothesized that transplantation of allogeneic MSCs may be a potential therapeutic approach for SLE. We have found that transplantation of allogeneic/xenogeneic BM-MSCs is capable of reconstructing the bone marrow osteoblastic niche and is more effective in reversing multi-organ dysfunction than immunosuppressive agents such as cyclophosphamide.[27,28] Currently, BM-MSCs represent the major source of MSCs for cell therapy. However, aspiration of BM-MSCs is invasive, and the population and differentiation potential of BM-MSCs decrease significantly with age.[29] In contrast, umbilical cord-MSCs (UC-MSCs) may be collected without causing pain to the donors, and these cells have greater proliferative potential. Therefore, UC-MSCs should be considered as a promising alternative to BM-MSCs as the source of MSCs for allogeneic transplantation.[30]

The aims of the present study were to investigate whether UC-MSCs have therapeutic effects in MRL/lpr mice, and the possible mechanisms underlying this treatment. Our results indicated that UC-MSCs are effective in preventing the development of lupus-like nephritis in MLR/lpr mice, which may provide the impetus to use UC-MSCs in cell-mediated therapy for the management of LN in SLE patients.

Materials and methods

Mice

Fifty-six female MRL/lpr mice (6 weeks old), weighing 20.5 ± 0.6 g (mean ± SD), were purchased from Shanghai SLAC Laboratory Animal Institute Co. Ltd. The mice were maintained in a specific pathogen-free animal facility of the Affiliated Drum Tower Hospital of Nanjing University Medical School. The MRL/lpr mice were randomly divided into the following five groups (eight mice in each group): group 1 mice receiving transplantation of 1×10^6 UC-MSCs (UC-MSCT) once at 18 weeks of age; group 2 mice receiving multi-transplantation of 1×10^6 UC-MSCs (multi-UC-MSCT) at three consecutive weeks (18, 19, and 20 weeks of age); group 3 receiving transplantation of 1×10^6 BALB/C mice BM-MSCs (BM-MSCT) once at 18 weeks of age; group 4 mice receiving 0.5 ml saline at 18 weeks of age (control); and group 5 mice receiving 1×10^6 UC non-adherent cells at 18 weeks of age (Figure 1A). The experimental protocols conformed to the animal care guidelines of the China Physiologic Society and were approved by our Institutional Animal Research Committee.

MSCs culture

Mouse BM-MSCs were isolated and cultured as described previously.[28] Umbilical cord (UC) was obtained from the Gynecology Department at Affiliated Drum Tower Hospital of Nanjing University Medical School. Tissue collection for this study was approved by The Affiliated Drum Tower Hospital Ethics Committee and informed consent was obtained from newborns' parents. The tissue was minced into 1–2 mm^3 pieces, and the minced tissue was incubated with 0.075% collagenase type II (Sigma, St Louis, MO, USA) for

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30 min and then with 0.125% trypsin (Gibco, Grand Island, NY, USA) for 30 min with gentle agitation at 37°C. The digested mixture was then passed through a 100 μm filter to obtain cell suspensions. These cells were used for group 5 mice. Cells from UC or bone marrow (BM) were plated at a density of 1×10^6 cells/cm^2 in non-coated T-25 cell culture flasks (Becton Dickinson, San Jose, CA, USA). Growth medium consisted of Dulbecco's modified Eagle's medium with low glucose (Gibco) and 5% fetal bovine serum (FBS, HyClone, Logan, UT, USA), supplemented with 10 ng/ml vascular endothelial growth factor (Sigma), 10 ng/ml epidermal growth factor (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), and 2 mmol/L glutamine (Gibco). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The medium was replaced and non-adherent cells were removed after 3 days. The medium was changed twice weekly thereafter. A cell monolayer formed within 2 weeks, consisting of homogeneous bipolar spindle-like cells in a whirlpool-like array. Flow cytometric analysis showed that the UC-derived cells were positive for CD29, CD44, CD105, and CD166, but negative for CD14, CD34, CD38, CD45, and HLA-DR. Once 60–80% confluence had been reached, adherent cells were re-plated at a density of 1×10^4/cm^2 in UC-MSCs growth medium (UC-GM) for expansion. After passage 3, cells were used for transplantation. Flow cytometric analysis was performed on passage 2.

Laboratory renal function assessments

To compare renal function between treated and untreated animals, mice urinary protein was measured every 2–3 weeks. Twenty-four-hour urine was collected using metabolism cages every 2–3 weeks. Urinary protein concentration was measured by Coomassie Brilliant Blue. Serum creatinine (Cr) was assessed in each group at the 29th week and anti-double-stranded DNA (dsDNA) antibodies were analyzed by enzyme-linked immunosorbent assay (ELISA; Shibayagi, Gunma, Japan).

Immunohistopathologic analysis

To assess pathologic kidney changes after MSCs transplantation, the kidneys were cut into small pieces and fixed in 10% formalin for 24 h at 4°C. Paraffin sections (4 μm) were stained with hematoxylin and eosin (HE) and periodic acid–Schiff (PAS). To detect immune complex deposits, cryostat sections (4 μm) were fixed in chilled acetone and stained with a fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-mouse IgG or IgM antibody (Organon Teknica, Scarborough, CA). The severity of glomerulonephritis was evaluated in a blinded manner by histologic examination of the sectioned kidneys. Results were expressed as the crescent formation rate. One hundred glomerular cross-sections (GCS) per kidney were examined. Crescent formation rate was defined as the number of glomeruli with crescent formation/GCS according to standard assay.[31] Two kidneys of each group were prepared for transmission electron microscopy. Kidneys were prefixed in 4% glutaraldehyde at 4°C for 60 min, washed with 0.1% sodium cacodylate buffer (pH 7.2–7.4) four times at 4°C for 15 min, stained with 1% osmic acid for 2 h, and poached in double distilled water for 10 min. The samples, attached to grids, were dehydrated in ethanol and dried. Ultrathin sections were cut using an ultramicrotome and stained with both uranyl acetate and lead citrate. Sections were observed under a JEM-1230 transmission electron microscope at an accelerating voltage of 80 kV by two pathologists, who were blinded to the treatments.

RNA isolation and real-time quantitative PCR

To investigate the production of HMGB-1, MCP-1 in kidney and Foxp3 in spleen after the MSCs treatment, total RNA was extracted from renal cortex cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The production of MCP-1 and HMGB-1 mRNA in kidney and Foxp3 mRNA in spleen was quantified by real-time quantitative polymerase chain reaction (PCR) using the TaqMan PCR MASTER MIX kit (Applied Biosystems, Foster City, CA). The relative production of each mRNA was determined and normalized to the expression of the internal housekeeping gene GAPDH. Primer and probe sequences are described as follows: MCP-1(103 bp): forward, 5′-AGAAACCAGCCA ACTC-3′, reverse, 5′-GCTACAGGCAGCA ACT-3′; HMGB-1 (359 bp): forward 5′-ATGTTCT GCTCCTTACC-3′, reverse 5′-AGTTTATCCG CTTTCC-3′; Foxp3 (391 bp): forward, 5′-GCTG GCAAATGGTGTCT-3′, reverse, 5′-CTCTGGG AATGTGCTGTT-3′; GAPDH (115 bp): forward, 5′-ATCGTGGAAGGGCTAATG-3′ and reverse, 5′-GGATGATGTTCTGGTGGG-3′.

Immunohistochemistry, western blot analysis and ELISA

To detect MCP-1 and HMGB-1 expression, kidneys were snap-frozen in optional cutting temperature solution (OCT) compound (Sakura, Osuka, Japan)

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and cut into 5-μm pieces. Sections were analyzed by the avidin–biotin–peroxidase method, using biotin-labeled goat anti-murine MCP-1 polyclonal antibody (Santa Cruz, CA) and HMGB-1 polyclonal antibody (Santa Cruz, CA). Preimmune biotin-labeled goat serum served as a negative control. Analysis with monoclonal antibody (mAb) against human nuclei (MAB1281, Chemicon International) was performed following the manufacturer's instructions to detect UC-MSCs in kidneys of mice treated with UC-MSCs. For western blot analysis, kidney homogenates were blotted with anti-MCP-1 (Santa Cruz, CA) and anti-mouse HMGB-1 antibodies (Santa Cruz, CA). Spleen homogenates were blotted with anti-Foxp3 (Sigma, St Louis, USA). Anti-actin antibody (Sigma, St Louis, USA) was used as a loading control. Horseradish peroxidase-conjugated antibodies were used as secondary antibodies. Band detection was conducted using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, USA). Serum and urinary MCP-1 were measured using ELISA. Briefly, plates were coated with hamster anti-mouse MCP-1 (BD Pharmingen) at 10 μg/ml in 0.1 M Na_2HPO_4 (pH 9.0) overnight at 4°C. After blocking, plates were incubated overnight with sample, then for 2 h at room temperature with 0.5 μg/ml biotinylated hamster anti-mouse MCP-1 (BD Pharmingen), followed by streptavidin–HRP (Chemicon International). Plates were developed using tetramethylbenzidine substrate and optical density was read at 450 nm.

CFSE labeling

To investigate the trafficking of UC-MSCs in vivo, UC-MSCs were detached using trypsin, resuspended at a concentration of 1×10^7 cells/ml in phosphate-buffered saline (PBS), and then labeled with 2 μM/ml CFSE (Molecular Probes, Oregon, USA) for 10 min at 37°C before being washed with PBS and put back into culture for monitoring of fluorescent staining retention over a period of 2 weeks.

In vivo migration assay

To trace the migration of transplanted cells in vivo, labeled cells were then injected into the lateral tail vein of 16 MRL/lpr mice (10^6 cells per animal). Animals were killed either 24 h or 1 week later. To detect CFSE-labeled cells, cryostat sections of spleen, lungs, liver and kidney (4 μm) were fixed in chilled acetone.

$CD4^+$ T-cell isolation and co-culture

To determine the role of Treg cells in the MSCs treatment, spleens were collected from the mice and single-cell suspensions were prepared by mechanical disruption in RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml of penicillin, 100 mg/ml of streptomycin, 1×nonessential amino acids, 1 mM sodium pyruvate, 2.5 mM b-mercaptoethanol and 2 mM L-glutamine. $CD4^+$ T cells were isolated by magnetic sorting with Dynabeads mouse $CD4^+$ beads followed by DETACHaBEAD Mouse $CD4^+$ (Dynal Biotech), according to the manufacturer's directions. Negatively selected cells contained on average 99% $CD4^+$ T cells, as assessed by flow cytometric analysis with a CD4 mAb. All cell cultures were performed in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), in the absence or presence of the following stimuli: interleukin 10 (IL-10; 10 ng/ml, Sigma), anti-transforming growth factor-β neutralization antibody (anti-TGF-β; 10 μg/ml, Sigma).

Flow cytometric analysis

Single cell suspensions were obtained from the spleen. Resuspended cells were layered over 1.077 g/ml Ficoll solution (TBD, Tianjin, China) and centrifuged at 600 g for 20 min at room temperature. The peripheral blood mononuclear cells (PBMC) were collected at the interface, washed with PBS, resuspended with 100 μl staining buffer (0.5% bovine serum albumin, 0.04% EDTA, 0.05% sodium azide in PBS), divided into two aliquots (one for detection and another for hemotype control), and stained with antihuman fluorescein isothiocyanate (FITC)-conjugated CD4/allophycocyanin (APC)-conjugated CD25 (clone PRA-T4/BC96, eBioscience, USA) for 30 min at 4°C in the dark. Stained cells were then washed with staining buffer. For Foxp3 expression, 1 ml fixation/permeabilization concentrate/diluent was added to each tube. Cells were then incubated for 60 min at 4°C in the dark, washed with permeabilization buffer and blotted with intracellular phycoerythrin (PE)-conjugated anti-mouse Foxp3 (eBioscience, USA). PE-conjugated rat IgG2a isotype (clone eBR2a, eBioscience, USA) was used as control. After incubation for 30 min at 4°C in the dark, stained cells were washed twice with diluted permeabilization buffer and fixed with 1% paraformaldehyde in PBS. The data were acquired on a FACSCalibur (BD

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Immunocytometry Systems) and analyzed using CellQuest software.

Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). SPSS 11.0 software was used for statistical analysis. The single-factor analysis of variance (ANOVA) was used for the comparison among multiple sample means. We considered $p < 0.05$ as statistically significant.

Results

UC-MSCs transplantation alleviates LN in MRL/lpr mice

In MRL/lpr mice, Fas-mediated apoptosis of activated lymphocytes was severely impaired, and T-cell-dependent production of autoantibodies resulted in immune complex-mediated glomerulonephritis and vasculitis.[32,33] In the present study, two and six mice died in the control and UC-non-adherent cells groups, respectively. It has been reported that autoantibodies play a crucial role in multiple organ impairment in SLE patients. Consistent with human findings, MRL/lpr mice showed a remarkable increase in circulating autoantibodies, specifically anti-dsDNA. Multi-UC-MSCT mice benefited from a significant reduction in serum levels of anti-dsDNA antibody compared with that in control animals. In addition, a moderately decreased level of anti-dsDNA antibody was observed in UC-MSCT and BM-MSCT, but there was no significant difference from control groups (Figure 1B). MRL/lpr mice showed renal disorders such as nephritis with glomerular basal membrane inflammation, mesangial cell overgrowth, and deposition of complement component 3 (C3), IgG and IgM. We found that all the treatment groups showed much less proteinuria, lower blood urea nitrogen (BUN) and lower Cr in comparison with control mice. Interestingly, the levels of Cr in multi-UC-MSCT mice were significantly decreased in comparison with those in UC-MSCT and BM-MSCT mice (Figure 1B). This experimental evidence indicated that UC-MSCT treatment was capable of restoring renal function. Further histological analysis demonstrated that the kidneys from the control mice showed typical glomerulonephritis with the above-mentioned characteristics. Electron-dense deposits in both mesangial and subepithelial areas were readily seen in control kidneys. Interestingly, both UC-MSCT and BM-MSCT were able to improve renal disorders, specifically restoring kidney glomerular structure, and reducing glomerular IgG and IgM deposition. No, or very sparse, electron-dense deposits were found in the treatment groups (Figure 1C). These morphological observations were further confirmed by the crescent formation rate. The degree of crescent formation was significantly different among the treatment and control groups. In addition, the degree of crescent formation in the multi-UC-MSCT group was significantly lower than that in the UC-MSCT and BM-MSCT groups (Figure 1D). These findings suggest that UC-MSCT is a superior therapeutic approach for treating nephritis in MRL/lpr mice and is capable of restoring renal function. Multi-infusion of UC-MSCs may enhance their effects.

UC-MSCs transplantation decreases the expression of MCP-1 and HMGB-1 in kidney of MRL/lpr mice

Recent studies have demonstrated that overproduction of proinflammatory cytokines such as MCP-1[1–4] and HMGB-1[34] play an important role in the pathogenesis of LN in humans and animal models. We found that the levels of serum MCP-1 in multi-UC-MSCT mice were significantly decreased in comparison with the control group, and urine MCP-1 showed a similar pattern (Figure 2A). Levels of MCP-1 protein expression in all the treatment mice were significantly decreased in comparison with the control group ($p < 0.05$). Expression of MCP-1 protein in the multi-UC-MSCT group was significantly lower than in UC-MSCT and BM-MSCT mice (Figure 2B, $p < 0.05$). A similar pattern was revealed in MCP-1 mRNA expression in the kidney (Figure 2C). By immunochemical staining, MCP-1 was primarily located in glomerular mesangium, renal interstitium and renal tubules. The positive staining was located at the edge and lumen of renal tubules. The staining in the control group was more intense than that in the treatment group (Figure 2D). In addition, we found that the expression of HMGB-1 protein in all the treatment mice was significantly lower than in controls (Figure 2E, $p < 0.05$). The differences in mRNA expression corresponded well with protein expression (Figure 2F). Immunohistochemical staining for HMGB-1 showed marked intense staining in the control kidneys. This positive staining was much weaker among all the treatment groups (Figure 2G). These results indicate that transplanting UC-MSCs is effective in the treatment of LN, possibly by inhibiting MCP-1 and HMGB-1 expression.

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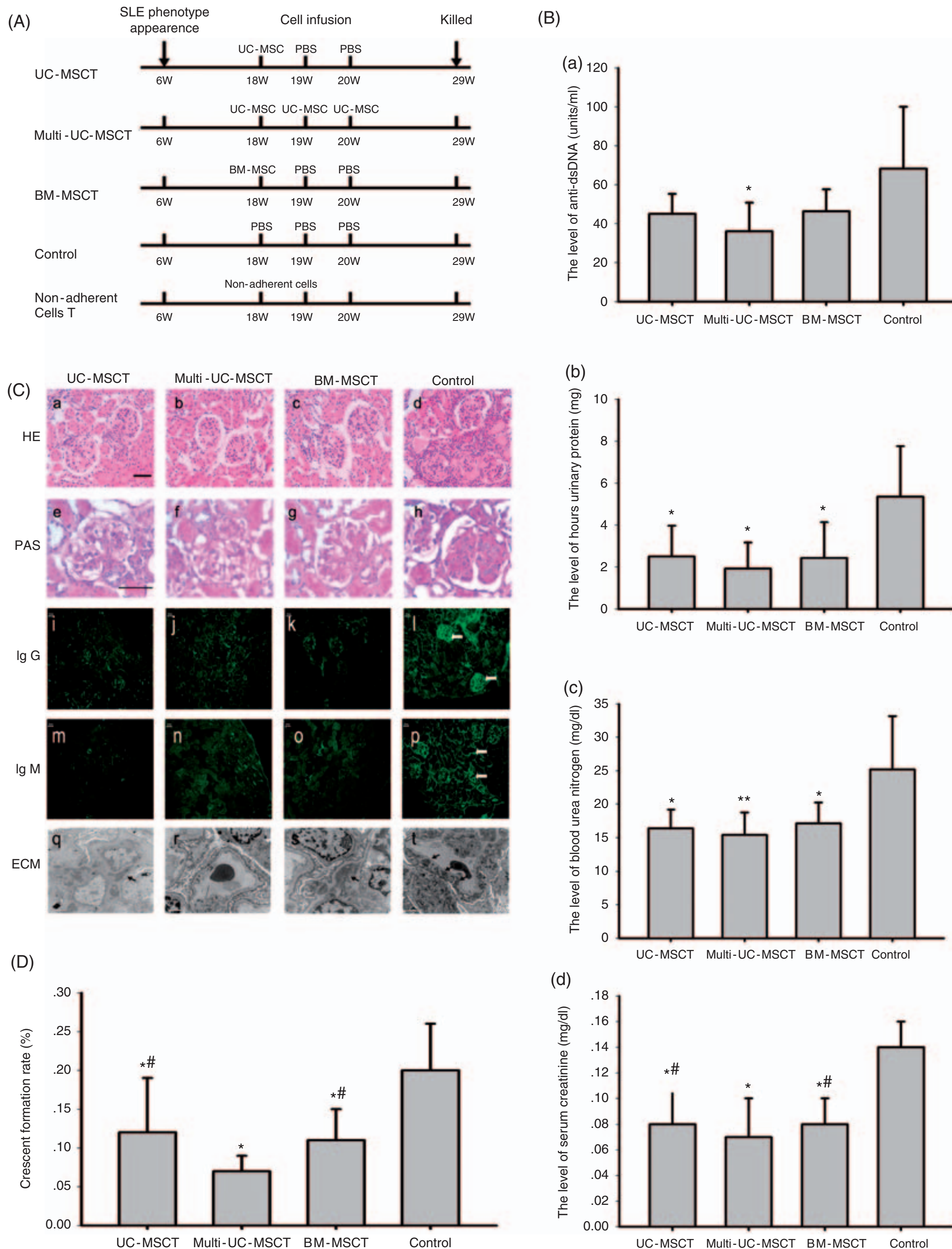

Figure 1 UC-MSCT reduced levels of anti-dsDNA antibodies and improved renal function in MRL/lpr mice. (A) The scheme of UC-MSCT, multi-UC-MSCT, and BM-MSCT treatment procedures. (B) There was a significant difference between the levels of anti-dsDNA antibody in multi-UC-MSCT and that in control (*$p < 0.05$ vs control) (a). All treatments (UC-MSCT, $n = 8$; multi-UC-MSCT, $n = 8$; BM-MSCT, $n = 8$) significantly reduced urine protein (b), BUN (c), and Cr (d) levels (mean ± SD) in MRL/lpr mice compared with control mice ($n = 6$). In addition, multi-UC-MSCT appeared to significantly decrease the level of Cr compared with that in the UC-MSCT and BM-MSCT groups (*$p < 0.05$ vs control, **$p < 0.01$ vs control, $^{\#}p < 0.05$ vs multi-UC-MSCT). Three repeated tests per group showed similar results. (C) UC-MSCT and multi-UC-MSCT, as well as BM-MSCT, reduced basal membrane disorder and mesangium cell overgrowth in glomerulus, infiltration of inflammatory cells, and crescent formation (top row, H&E staining; second row, PAS staining). Immunofluorescence showed that all the treatments were able to reduce deposition of IgG and IgM (third and fourth rows). By electron microscopy (arrow), no, or very sparse, electron-dense deposits were found in the treated mice (bottom row). Bar = 20 µm. (D) All the treatments reduced the crescent formation rate. The degree of crescent formation in all the treatment groups was significantly lower than in the control group. In addition, the degree of crescent formation in the multi-UC-MSCT group was significantly lower than in the UC-MSCT and BM-MSCT groups (*$p < 0.05$ vs control, $^{\#}p < 0.05$ vs multi-UC-MSCT).

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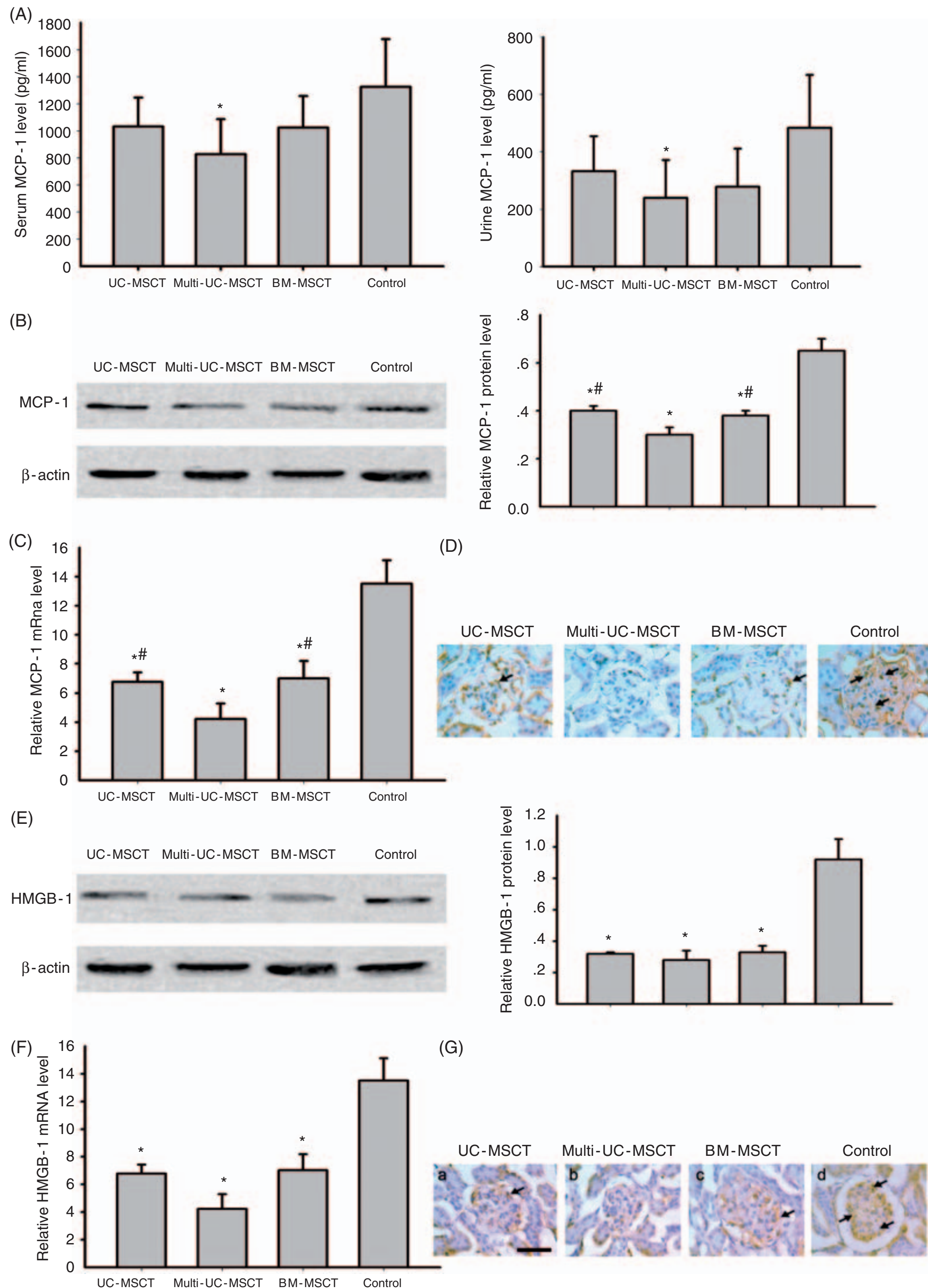

Figure 2 UC-MSCs treatment decreased the expression of MCP-1 and HMGB-1. (A) ELISA confirmed decreased levels of MCP-1 in serum and urine in MRL/lpr mice following multi-UC-MSCs treatment compared with control; however, UC-MSCT and BM-MSCT showed no changes (*$p < 0.05$ vs control). (B,C) Western blot (B) and real-time PCR (C) analyses showed that all the treatment groups showed a significant decrease in the expression of MCP-1. In addition, the expression of MCP-1 in kidney of multi-UC-MSCT mice was also significantly decreased compared with that in UC-MSCT and BM-MSCT mice (*$p < 0.05$ vs control, $^{\#}p < 0.05$ vs multi-UC-MSCT). β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls in RT-PCR and western blot, respectively. Three repeated tests per group showed similar results. (D) Immunohistochemical staining with anti-MCP-1 antibody indicated that the positive area (arrow) was more intense than that in the treatment group. Bar = 20 μm. (E,F) HMGB-1 protein (E) and mRNA expression (F) in control kidneys were significantly higher than in all the treatment groups (*$p < 0.05$ vs control). β-actin and GAPDH were used as loading controls in RT-PCR and western blot, respectively. Three repeated tests per group showed similar results. (G) Immunohistochemical staining for HMGB-1 showed marked intense staining in the control kidneys. This positive staining (arrow) was much weaker in all the treatment groups. Bar = 20 μm.

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UC-MSCs treatment increases the percentage of Treg in vivo and in vitro

It has been suggested that $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ T cells prevent autoimmunity. To investigate whether UC-MSCs affect Treg in MRL/lpr mice, we studied $Foxp3^{+}$ T cells in spleen of MRL/lpr mice. Flow cytometry demonstrated that $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ T cells in the spleen of MRL/lpr mice were significantly increased by UC-MSCs treatment, as compared with the control group. Likewise, BM-MSCT also increased $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ T cells in MRL/lpr mice (Figure 3A, Supplementary Information Figure S1). In vitro, purified $CD4^{+}$ T cells were incubated without or with UC-MSCs at a 1 : 1 ratio in the presence of IL-10 and anti-TGF-β. Both percentage of Treg and level of Foxp3 expression were significantly increased in the UC-MSC-treated group, in comparison with non-UC-MSC-treated controls. Also, the effect of UC-MSCs on $CD4^{+}$ T cells could be inhibited in the presence of IL-10 or anti-TGF-β (Figure 3B–D, Supplementary Information Figure S2). Thus, UC-MSCs upregulate the Treg, a population at least partially decreased in lupus due to diminished secretion of IL-10 and raised secretion of TGF-β. This evidence indicated that UC-MSCs attenuated LN in MRL/lpr mice, possibly through the induction of Treg.

UC-MSCs infusion homing to several tissues in MRL/lpr mice

Numerous studies have suggested that UC-MSCs are of potential therapeutic value. Transplantation of ex vivo expanded allogeneic (allo-)MSCs can repair infarcted heart and brain[35,36] and enhance wound healing in animals.[37] In the present study, 24 h after transfusion, the highest frequency of UC-MSCs was found in the lungs and kidneys; CFSE-positive cells were also detected in the liver, spleen and lymph nodes. Homing of UC-MSCs analyzed 1 week later was similar to that at 24 h, while there was considerable variation between animals in CFSE-positive cells in the lymph nodes and spleen. Few CFSE-positive cells were found in the heart either 1 day or 1 week after transfusion (Figure 4A). These findings were supported by MAB1281 analysis. MAB1281-positive cells identified in murine kidney were also stained positive with a human specific anti-HLA class I mAb in MRL/lpr mice 11 weeks after transplantation (Figure 4B). The in vivo studies showed that UC-MSCs can home to damaged areas and might subsequently contribute to the repairing of damaged tissue.

Discussion

In the present study, we have demonstrated that transfusions of xenogeneic UC-MSCs significantly attenuate the severity of glomerulonephritis in MRL/lpr mice. There were significant differences in the urinary proteins and serum creatinine levels between the treatment and control groups as well as the crescent formation rate in lupus mice. Light microscopic examination of the kidney tissues showed that the improvement of renal pathology correlates well with reduced expansion of extracellular matrix (ECM) of the glomeruli and the number of infiltrating leukocytes. Serum anti-dsDNA antibodies are well known as a signature antibody of active SLE. Our results showed that the serum anti-dsDNA antibody levels were significantly lower in MRL/lpr mice after treatment with infusion of UC-MSCs. It is of interest that three transfusions provided more significant reduction in the above-mentioned disease activity parameters.

MCP-1, a potent chemoattractant of monocytes and T cells, is secreted by many intrinsic renal cells, including endothelial, mesangial, tubular epithelial and interstitial cells, in response to stimulation with proinflammatory cytokines and immune complexes (ICs).[38] Overexpression of MCP-1 in renal tissue parallels mononuclear cell accumulation. Anti-MCP-1 gene therapy is specifically effective for local inflammation.[39] Perez *et al*. found that chemokine-induced monocyte infiltration preceded the occurrence of proteinuria and kidney damage in MRL /lpr mice.[4] Our results indicated that transplantation of UC-MSCs decreased the expression of MCP-1 in kidney of MRL/lpr mice. The improved outcomes could therefore be explained, at least partially, by the inhibitory effects on the MCP-1 signaling pathways in the kidneys. It is noteworthy that MCP-1 expression is lower in mice with multiple transfusions than in the single transfusion group. This may indicate that the treatments are effective in reversing the expression of this chemokine.

HMGB proteins are abundant non-histone DNA-binding proteins that help to maintain nucleosome structure and regulate gene transcription.[40] They are ubiquitously present in the nuclei of mammalian cells and are highly conserved among species. In addition to its DNA-binding ability, HMGB-1 can stimulate an inflammatory cytokine response upon passive release into the extracellular space from necrotic cells, or following active release from activated monocytes and

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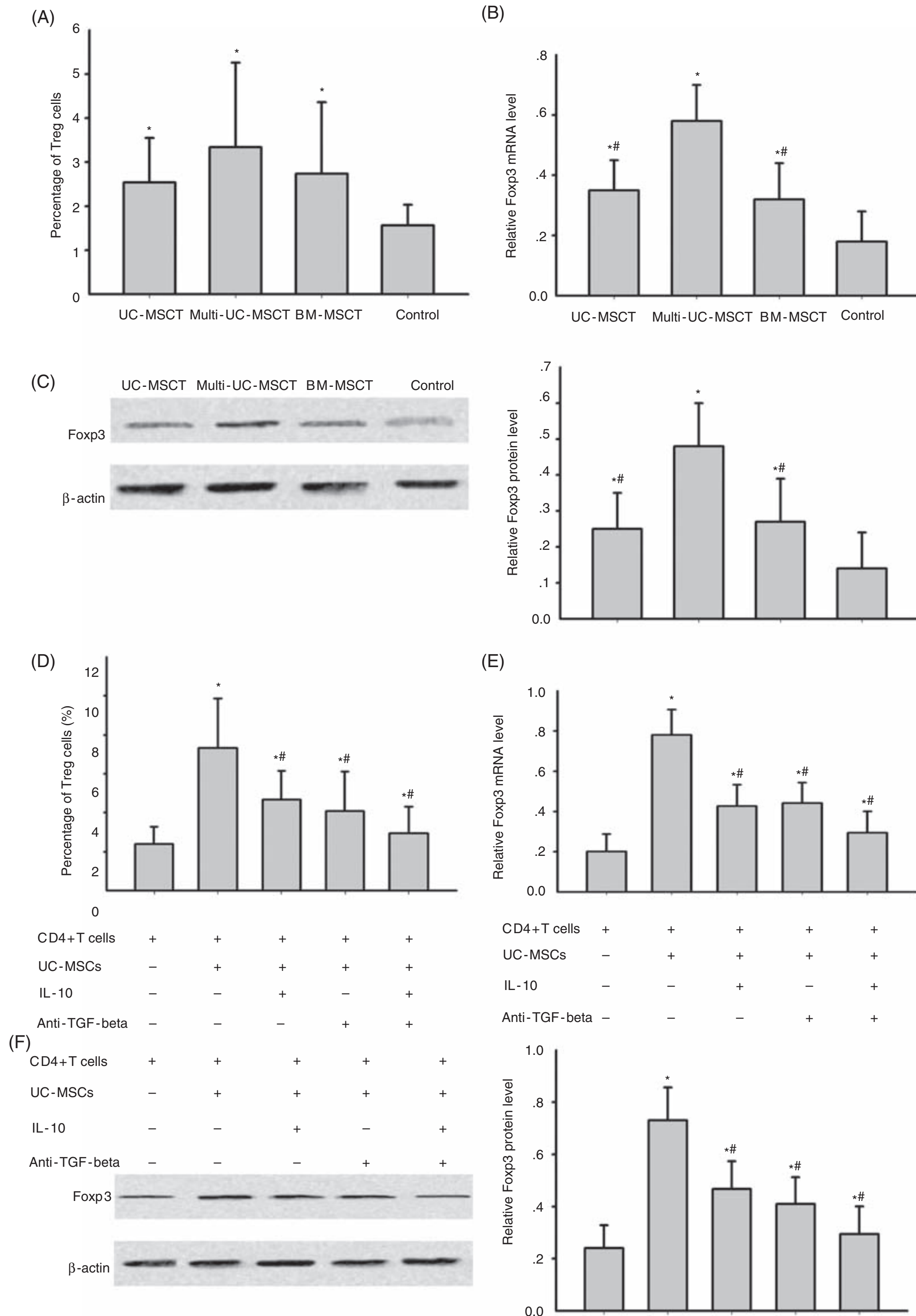

Figure 3 UC-MSCs treatment increased $CD4^+$ $CD25^+$ $Foxp3^+$ Treg induction. (A) Flow cytometry revealed that all the treatment groups had significantly increased levels of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg in the spleen compared with the control group (*$p < 0.05$ vs control). The results were representative of three independent experiments. (B) Flow cytometry assay showed that UC-MSCs co-culture upregulated frequencies of Treg cells ratio in vitro, and that the effect of UC-MSCs on $CD4^+$ T cells could be inhibited by IL-10 or anti-TGF-β. The results were representative of three independent experiments. (C,D) Real-time PCR (C) and western blot (D) analyses showed that the expression of Foxp3 at mRNA and protein levels was upregulated when co-cultured with UC-MSCs at 1 : 1 ratio for 3 days. The effect can be inhibited by IL-10 and anti-TGF-β. GAPDH and β-actin were used as loading controls in RT-PCR and western blot, respectively (*$p < 0.05$ vs $CD4^+$T cells, $^{\#}p < 0.05$ vs $CD4^+$ T cells plus UC-MSCs). The results were representative of three independent experiments.

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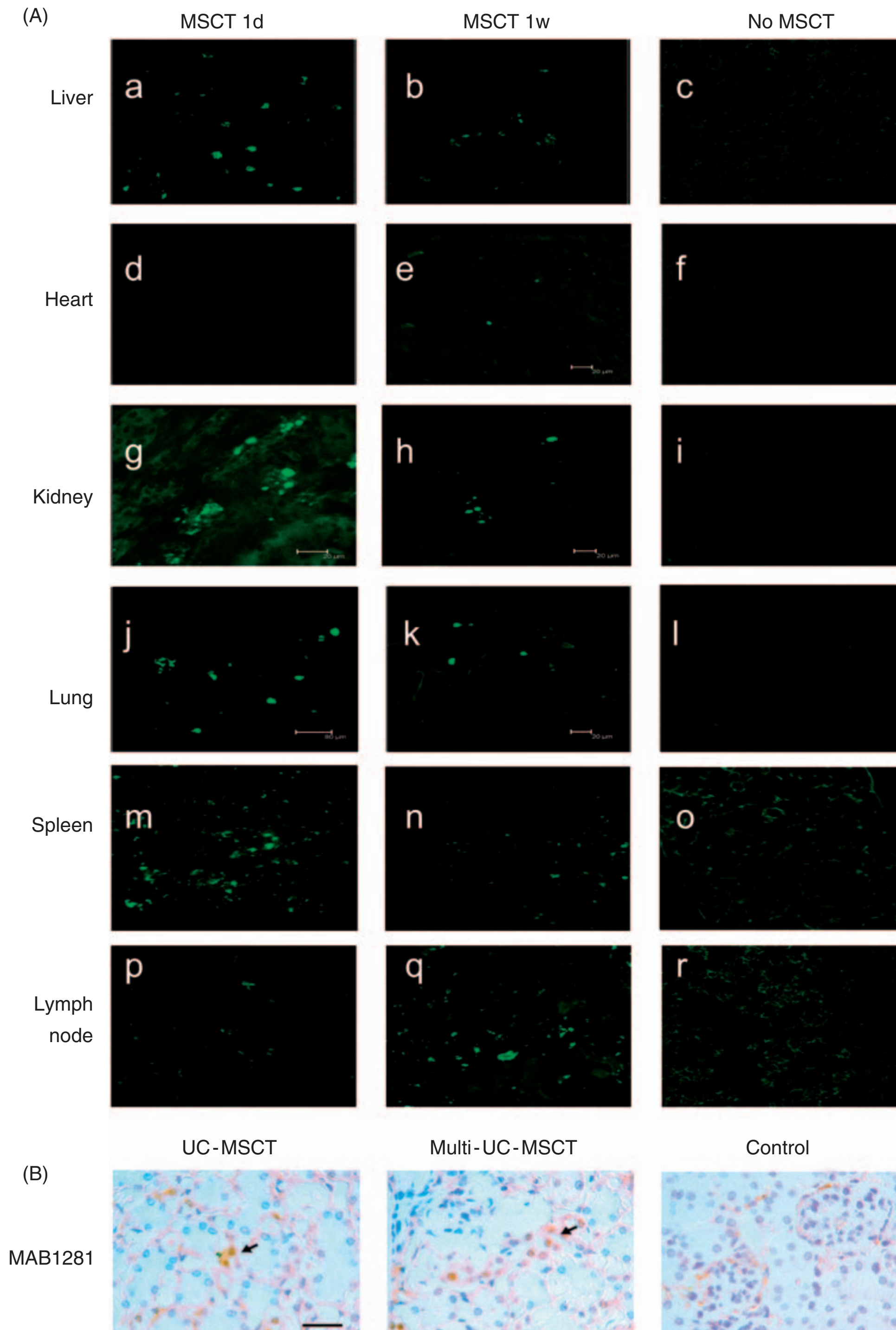

Figure 4 UC-MSCs homed to several tissues in MRL/lpr mice. (A) Twenty-four hours after transfusion, high frequencies of UC-MSCs were found in the lungs and kidneys, lymph nodes, spleen and liver. Fewer CFSE-positive UC-MSCs were found in the heart. Homing of UC-MSCs analyzed 1 week later was similar to that at 24 h. (B) MAB1281-positive cells (arrow) identified in murine kidneys were also stained positive with a human-specific anti-HLA class I monoclonal antibody in MRL/lpr mice 11 weeks after transplantation.

macrophages.[5,41] During its secretion, HMGB-1 exits the nucleus, is transported through the cytoplasm, and is then actively released into the extracellular space.[42] HMGB-1 can also be passively released from the nuclei of necrotic or damaged cells. However, cells undergoing apoptosis are poor HMGB-1 secretors.[43] HMGB-1 has been shown to act as an endogenous immune adjuvant

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by activating antigen-presenting cells (including dendritic cells and macrophages), through the receptor of advanced glycation end products (RAGE) and possibly toll-like receptor 2 and 4 mechanisms.[44,45] Interestingly, it was recently shown that HMGB-1 and RAGE mediated TLR9-dependent activation of plasmacytoid dendrite cells by DNA-containing ICs.[38] Importantly, nephritogenic anti-dsDNA-inducing Th cell lines derived from patients with active lupus nephritis proliferate in response to HMGB-1. Anti-DNA antibody in these Th cell lines increased 250-fold when co-cultured with autologous B cells.[46] Thus, autoimmune Th cells can provide help to B cells that process and present HMGB-1 peptides. Anti-HMGB-1 antibody was found in SLE patients.[47] Popovic and his colleagues have found high amounts of extracellular HMGB-1 in skin lesions of lupus.[48] Deocharan *et al.* found that immunization of non-autoimmune mice with α-actinin induced strong anti-nuclear antibody (ANA) response, particularly against chromatin. Furthermore, kidney glomerular IgG deposition and proteinuria were present in α-actinin-immunized mice. The cross-reactive chromatin targets were determined to be HMGB-1 protein and heat shock protein 70, both of which are known as antigenic targets in SLE.[49] In the present study, we showed that the expression of HMGB-1 was significantly reduced in all the treated mice in comparison with that in control animals. Therefore, downregulation of HMGB-1 expression may be one of the mechanisms involved in the treatment of MRL/lpr mice by UC-MSCs.

Tregs play an important role in the prevention of autoimmunity. It has been demonstrated that they modulate the severity of SLE. Low numbers or functional defects in $CD4^+$ $CD25^+$ T cells have been found in SLE.[11,50] Natural $CD4^+$ $CD25^+$ Treg express the transcription factor Foxp3, which controls their development and function. Functional defects in the Foxp3 gene lead to the absence of Treg generation.[9] The immune cell effector functions in response to lymphopenia, infection and autoimmune reactivity are also under the strict control of Treg.[9] We have found higher percentages of $CD4^+$ $Foxp3^+$ T cells in the spleen of UC-MSCs-treated mice. In vitro UC-MSCs upregulate the Treg population. Treg is decreased in lupus, at least partially due to the diminished secretion of IL-10 and raised secretion of TGF-β. A previous study has also found that treatment with autologous BM-MSCs increased $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in two SLE patients, but without decreasing the SLEDAI.[51] These results may indicate some intrinsic functional defects of BM-MSCs from SLE patients, as we have previously reported,[26] which may reduce or even abolish the therapeutic effect of MSCs infusions in SLE patients with autologous transplantation. Alternatively, it is plausible to reason that our observed effects of UC-MSCs treatment on inhibiting the development of lupus like nephritis in MLR/lpr mice may be mediated by other immune cells or functions in addition to Treg cells. Recently, a study by Traggiai *et al.* reported, though their data were not shown, that NZB/NZW F1 lupus-prone mice injected with MSCs had no improvement in lupus nephritis.[52] Future studies are warranted to determine whether differences in animal lines or dosages of MSCs treatments may account for the discrepancies observed in the effects of MSCs on the development and progression of lupus nephritis. Intriguingly, MSCs have been shown to either suppress B-cell proliferation and antibody production or induce B-cell proliferation into plasma cells.[52] Although our current data favor a role of UC-MSCs in inhibiting B-cell functions, the effects of UC-MSCs on B-cell activation and antibody production are currently being investigated in our laboratory.

In summary, our study has shown that infusion of UC-MSCs exerts a similar therapeutic effect to BM-MSCs in treating LN in MRL/lpr mice without obvious major side effects. UC-MSCs were able to decrease levels of 24-h urine protein, serum creatinine, and anti-dsDNA antibody, which also improved renal pathological injury, reduced crescent formation, and reduced MCP-1 and HMGB-1 expression in MRL/lpr mice. The results demonstrate that UC-MSCs could effectively prevent the development of lupus nephritis. However, it remains to be determined whether UC-MSCs transfusions will reverse progression of established lupus nephritis. Nevertheless, our findings provide an impetus for further investigations of the treatment of SLE with allogeneic MSCs readily available from umbilical cords.

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References

1 Marks SD, Williams SJ, Tullus K, Sebire N. Glomerular expression of monocyte chemoattractant protein-1 is predictive of poor renal prognosis in pediatric lupus nephritis. *Nephrol Dial Transplant* 2008; 23: 3521–3526.
2 Wada T, Yokoyama H, Su SB, *et al*. Monitoring urinary levels of monocyte chemotactic and activating factor reflects disease activity of lupus nephritis. *Kidney Int* 1996; 49: 761–767.
3 Tesch GH, Maifert S, Schwarting A, Rollins BJ, Kelley VR. Monocyte chemoattractant protein 1-dependent leukocytic infiltrates are responsible for autoimmune disease in MRL-Fas(lpr) mice. *J Exp Med* 1999; 190: 1813–1824.
4 Perez de Lema G, Maier H, Nieto E, *et al*. Chemokine expression precedes inflammatory cell infiltration and chemokine receptor and cytokine expression during the initiation of murine lupus nephritis. *J Am Soc Nephrol* 2001; 12: 1369–1382.
5 Andersson U, Wang H, Palmblad K, *et al*. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med* 2000; 192: 565–570.
6 Hayashi A, Nagafuchi H, Ito I, Hirota K, Yoshida M, Ozaki S. Lupus antibodies to the HMGB1 chromosomal protein: epitope mapping and association with disease activity. *Mod Rheumatol* 2009; 19: 283–292.
7 Hoffman RW. T cells in the pathogenesis of systemic lupus erythematosus. *Front Biosci* 2001; 6: D1369–1378.
8 Chan OT, Madaio MP, Shlomchik MJ. The central and multiple roles of B cells in lupus pathogenesis. *Immunol Rev* 1999; 169: 107–121.
9 Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; 22: 531–562.
10 Hsu WT, Suen JL, Chiang BL. The role of CD4CD25 T cells in autoantibody production in murine lupus. *Clin Exp Immunol* 2006; 145: 513–519.
11 Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient CD4+CD25 high T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol* 2007; 178: 2579–2588.
12 Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation* 1974; 17: 331–340.
13 Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 1988; 136: 42–60.
14 Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997; 276: 71–74.
15 Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* 2003; 57: 11–20.
16 Di Nicola M, Carlo-Stella C, Magni M, *et al*. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; 99: 3838–3843.
17 Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2005; 105: 2821–2827.
18 Krampera M, Glennie S, Dyson J, *et al*. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003; 101: 3722–3729.
19 Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 2003; 75: 389–397.
20 Corcione A, Benvenuto F, Ferretti E, *et al*. Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006; 107: 367–372.
21 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; 105: 1815–1822.
22 Le Blanc K, Frassoni F, Ball L, *et al*. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008; 371: 1579–1586.
23 Chen X, Armstrong MA, Li G. Mesenchymal stem cells in immunoregulation. *Immunol Cell Biol* 2006; 84: 413–421.
24 Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* 2008; 3: e1886.
25 Kunter U, Rong S, Djuric Z, *et al*. Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. *J Am Soc Nephrol* 2006; 17: 2202–2212.
26 Sun L, Zhang H, Feng X, Hou Y, Lu L, Fan L. Abnormality of bone marrow-derived stem cells in patients with systemic lupus erythematosus. *Lupus* 2007; 16: 121–128.
27 Zhou K, Zhang H, Jin O, *et al*. Transplantation of human bone marrow mesenchymal stem cell ameliorates the autoimmune pathogenesis in MRL/lpr mice. *Cell Mol Immunol* 2008; 5: 417–424.
28 Sun L, Akiyama K, Zhang H, *et al*. Mesenchymal stem cell transplantation reverses multiorgan dysfunction in systemic lupus erythematosus mice and humans. *Stem Cells* 2009; 27: 1421–1432.
29 Rao MS, Mattson MP. Stem cells and aging: expanding the possibilities. *Mech Ageing Dev* 2001; 122: 713–734.
30 Lu LL, Liu YJ, Yang SG, *et al*. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica* 2006; 91: 1017–1026.
31 Sadanaga A, Nakashima H, Masutani K, *et al*. Amelioration of autoimmune nephritis by imatinib in MRL/lpr mice. *Arthritis Rheum* 2005; 52: 3987–3996.
32 Van Parijs L, Abbas AK. Role of Fas-mediated cell death in the regulation of immune responses. *Curr Opin Immunol* 1996; 8: 355–361.
33 Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Adv Immunol* 1985; 37: 269–390.
34 Wang H, Yang H, Czura CJ, Sama AE, Tracey KJ. HMGB1 as a late mediator of lethal systemic inflammation. *Am J Respir Crit Care Med* 2001; 164: 1768–1773.
35 Amado LC, Saliaris AP, Schuleri KH, *et al*. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci U S A* 2005; 102: 11474–11479.
36 Li Y, Chen J, Zhang CL, *et al*. Gliosis and brain remodeling after treatment of stroke in rats with marrow stromal cells. *Glia* 2005; 49: 407–417.
37 Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 2007; 25: 2648–2659.
38 Tian J, Avalos AM, Mao SY, *et al*. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol* 2007; 8: 487–496.
39 Shimizu S, Nakashima H, Masutani K, *et al*. Anti-monocyte chemoattractant protein-1 gene therapy attenuates nephritis in MRL/lpr mice. *Rheumatology (Oxford)* 2004; 43: 1121–1128.
40 Yamada S, Maruyama I. HMGB1, a novel inflammatory cytokine. *Clin Chim Acta* 2007; 375: 36–42.
41 Rovere-Querini P, Capobianco A, Scaffidi P, *et al*. HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Rep* 2004; 5: 825–830.
42 Gardella S, Andrei C, Ferrera D, *et al*. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep* 2002; 3: 995–1001.

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43 Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; 418: 191–195.
44 Taguchi A, Blood DC, del Toro G, *et al*. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature* 2000; 405: 354–360.
45 Park JS, Gamboni-Robertson F, He Q, *et al*. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol* 2006; 290: C917–924.
46 Desai-Mehta A, Mao C, Rajagopalan S, Robinson T, Datta SK. Structure and specificity of T cell receptors expressed by potentially pathogenic anti-DNA autoantibody-inducing T cells in human lupus. *J Clin Invest* 1995; 95: 531–541.
47 Uesugi H, Ozaki S, Sobajima J, *et al*. Prevalence and characterization of novel pANCA, antibodies to the high mobility group non-histone chromosomal proteins HMG1 and HMG2, in systemic rheumatic diseases. *J Rheumatol* 1998; 25: 703–709.
48 Popovic K, Ek M, Espinosa A, *et al*. Increased expression of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in skin lesions of patients with lupus erythematosus. *Arthritis Rheum* 2005; 52: 3639–3645.
49 Deocharan B, Zhou Z, Antar K, *et al*. Alpha-actinin immunization elicits anti-chromatin autoimmunity in nonautoimmune mice. *J Immunol* 2007; 179: 1313–1321.
50 Crispin JC, Martinez A, Alcocer-Varela J. Quantification of regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun* 2003; 21: 273–276.
51 Carrion F, Nova E, Ruiz C, *et al*. Autologous mesenchymal stem cell treatment increased T regulatory cells with no effect on disease activity in two systemic lupus erythematosus patients. *Lupus* 2009; 19: 317–322.
52 Traggiai E, Volpi S, Schena F, *et al*. Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. *Stem Cells* 2008; 26: 562–569.

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