Original Article

Transplantation of umbilical cord mesenchymal stem cells alleviates pneumonitis of MRL/lpr mice

Yanju Zhang[1*], Yunfei Xia[2*], Songshi Ni[1*], Zhifeng Gu[2*], Hua Liu[1]

[1]Department of Respiratory Medicine, [2]Department of Rheumatology Medicine, Affiliated Hospital of Nantong University, Nantong 226001, China
*These authors contributed equally to this paper.
Corresponding to: Hua Liu. Department of Respiratory Medicine, Affiliated Hospital of Nantong University, Nantong 226001, China. Email: ntuliuhua@126.com.

Objective: To investigate whether the umbilical cord mesenchymal stem cells (UC-MSCs) transplantation in the MRL/lpr mice has effect or not on their pneumonitis and the possible mechanisms underlying this treatment.

Methods: Twenty four 18-week-old MRL/lpr female mice were divided into three groups as following: the group 2 (UC-MSCT group) have been transplanted with 1×10^6 UC-MSCs through caudal vein, the group 3 (multi-UC-MSCT Group) have been transplanted with 1×10^6 UC-MSCs three times and the group 1 (control group) have been treated with 0.5 mL phosphate buffer saline (PBS) as control. The histopathology of the lung was observed. The pulmonary expression of high mobility group box protein-1 (HMGB-1) was measured by western blot and detected by quantitation real time polymerase chain reaction (PCR). Immunohistochemistry method was used to detect HMGB-1 expressions in pulmo.

Results: In comparision to control ground mice, UC-MSCs significantly reduced interstitial pneumonitis in the MRL/lpr mice. The lung peribronchiolar lesion index of UC-MSCT group (1.40±0.24) and multi-UC-MSCT group (1.02±0.29) were significantly decreased as compared to control group (1.95±0.35) ($P<0.01$). The perivascular lesion index of UC-MSCT group (1.20±0.18) and multi-UC-MSCT group (1.08±0.16) were also significantly reduced as compared to control group (1.56±0.32) ($P=0.018$, 0.002) and the lung alveolar areas lesion index of control group (1.72±0.34) was significantly increased as compared to UC-MSCT group (1.30±0.21) and multi-UC-MSCT group (1.05±0.15) ($P=0.011$, 0.000). The lung HMGB-1 protein in UC-MSCT group (0.32±0.04) and in multi-UC-MSCT group (0.28±0.06) were both significantly decreased as compared to that in control group (0.80±0.21) ($P<0.05$). The level of HMGB-1 mRNA in UC-MSCT group (4.68±0.37) and in multi-UC-MSCT group (4.35±0.10) lung were both significantly decreased as compared to those in control group (16.29±3.84) ($P<0.05$). In immunohistochemical staining lung sections, high expression of HMGB-1 was found mainly located in the cytoplasm and extracellular matrix of MRL/lpr mice pulmonary epithelial cells, the expression of HMGB-1 in UC-MSCT group and multi-UC-MSCT group was significantly decreased as compared to that in the control group.

Conclusions: These findings indicate that UC-MSCs have a therapeutic effect on systemic lupus erythematosus (SLE) pneumonitis, possibly by inhibiting HMGB-1 expression, which suggests a potential application of UC-MSCs in the treatment of human lupus.

Keywords: Umbilical cord mesenchymal stem cells (UC-MSCs); systemic lupus erythematosus (SLE); pneumonitis; high mobility group box protein-1 (HMGB-1)

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Introduction

Systemic lupus erythematosus (SLE) is a common and potentially fatal autoimmune disease characterized by multi-organ injuries including renal, pulmonary, cardiovascular, neural, musculoskeletal, and cutaneous involvements. SLE can affect any organ at any stage, during the course of the disease, but the lungs are relatively involved late (1). Lung involvement can be sometimes the presenting feature of SLE in the form of pleuritis, pleural effusion, lupus pneumonitis or interstitial lung disease (ILD). Once the lungs are involved, there are always some other organs involved, such as glomerulonephritis, which represent SLE highly active. Acute lupus pneumonitis (ALP) or chronic ILD without an accurate treatment may lead to hypoxic respiratory failure and cause death in final. In 1999, Wang *et al.* (2) found that high-mobility group box chromosomal protein 1 (HMGB-1) can be released into the extracellular and mediate inflammatory responses, which was considered to be an important inflammation mediator of endotoxemia and sepsis. Recent studies have demonstrated that HMGB-1, actively secreted by macrophage/monocytes under inflammatory stimuli (3), was found to act as a proinflammatory cytokine in SLE. The presence of anti-HMGB1 antibodies correlates with disease activity in SLE patients (4). The induction of this proinflammatory cytokines may play a pathogenic role in the development of pneumonitis in MRL/lpr mice. 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 (5-7). These cells have been shown to have immunosuppressive properties and to reduce inflammation (8-11). Human MSCs suppress lymphocyte alloreactivity *in vitro* in mixed lymphocyte cultures through human leukocyte antigen-independent mechanisms (8). Previous studies showed that MSCs could inhibit lymphocyte proliferation induced by a variety of mitogens (11-13). 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 (14-16). MSCs, which can produce important growth factors and cytokines, have a strong propensity to ameliorate tissue damage in response to injury and disease. Relevant to this investigation, Huang *et al.* demonstrated that BM-MSCs could be transplanted into lung tissues of rats, and transformed into type II alveolar cells and was shown to prevent the development of pulmonary fibrosis (17). Sun *et al.* have reported that MSCs in patients with SLE grew much slower and showed senescence behavior compared with those in normal control patients (18). Based on these findings, we hypothesized that transplantation of allogeneic MSCs may be a potential therapeutic approach for SLE. 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 (19). Compared to BM-MSCs, umbilical cord-MSCs (UC-MSCs) may be collected without causing pain to the donors, and these cells have greater proliferative potential. Therefore, for allogeneic transplantation, UC-MSCs should be more promising than BM-MSCs. We have also found that UC-MSCs transplantation is effective in preventing the development of lupus-like nephritis in MLR/lpr mice (20). Then, what about lupus pneumonitis? Weather UC-MSCs have effect on the other hazardous complication of SLE or not?

In this study, our results indicated that UC-MSCs can relieve the extent of pulmonary injury in MLR/lpr mice, which may provide a new feasible measure for the management of lupus pneumonitis in SLE patients.

Materials and methods

Mice

Twenty-four female MRL/lpr mice (six weeks old), weighing 20.4±0.5 g (mean ± standard deviation, 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 Hospital of Nantong University. The MRL/lpr mice were randomly divided into the following three groups (eight mice in each group): group 1 mice receiving 0.5 mL phosphate buffer saline (PBS) at 18 weeks of age (control); group 2 mice receiving transplantation of 1×10^6 UC-MSCs (UC-MSCT) once at 18 weeks of age; group 3 mice receiving multi-transplantation of 1×10^6 UC-MSCs (multi-UC-MSCT) at three consecutive weeks (18, 19, and 20 weeks of age); At 29 weeks of age, the mice were sacrificed and the lung

tissue was collected. 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

UC was obtained from the Gynecology Department at Affiliated Hospital of Nantong University. Tissue collection for this study was approved by The Affiliated 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 30 min and then with 0.125% trypsin (Gibco, Grand Island, NY, USA) for 30 min with gentle agitation at 37 ℃. Cells from UC 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 (GM) 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 mg/mL streptomycin (Sigma), and 2 mmol/L glutamine (Gibco). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 ℃. The medium was replaced after three days. The medium was changed twice weekly thereafter. A cell monolayer formed within two 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.

Histopathological analysis

To assess pathologic lung changes after MSCs transplantation, the left lungs were cut into small pieces and fixed in 10% formalin for 24 h at 4 ℃. Paraffin sections (4 mm) were stained with hematoxylin and eosin (HE) and Masson. The severity of airtube and vascellum injury was evaluated in a blinded manner by histologic examination of the sectioned lungs. Results were expressed according to the assay of Hasegawa *et al.* (21). The perivascular and peribronchiolar infiltrates were scored on the basis of histopathological findings: 0, normal; 1, less than three cell layers; 2, three to six cell layers; or 3, more than six layers. The index of perivascular lesion was indicated as the sum of all the scores per section divided by the number of all vessels per section. The index of peribronchiolar lesion was indicated as the sum of all the scores per section divided by the number of all bronchioli per section. The infiltrates in alveolar areas in high-power fields (×400 magnification) were scored as follows: 0, no infiltrating mononuclear cells; 1, less than 10 infiltrating cells; 2, less than 20 infiltrating cells; or 3, more than 20 infiltrating cells. The alveolar lesions index was indicated as the mean value of 20 random fields per section. The sections were evaluated by one of us, who was blinded to the treatment given.

RNA isolation and real-time quantitative PCR

To investigate the production of HMGB-1 in lung after the MSCs treatment, total RNA was extracted from pulmonary epithelial cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The production of HMGB-1 mRNA in lung was quantified by real-time quantitative polymerase chain reaction (PCR) using the TaqMan PCR MASTER MIX kit (Applied Biosystems, Foster City, CA, USA). The production of mRNA was determined and normalized to the expression of the internal housekeeping gene GAPDH. Primer and probe sequences are described as follows: HMGB-1 (359 bp): forward, 5'-ATGTTCTGCTCCTTACC-3', and reverse, 5'-AGTTTATCCGCTTTCC-3'.

Immunohistochemistry, western blot analysis

To detect HMGB-1 expression, lungs were snap-frozen in optional cutting temperature solution (OCT) compound (Sakura, Osuka, Japan) and cut into 5 μm pieces. Sections were analyzed by the avidin-biotin-peroxidase method, using biotin-labeled goat anti-murine HMGB-1 polyclonal antibody (Santa Cruz, CA, USA). 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, lung homogenates were blotted with anti-mouse HMGB-1 antibodies (Santa Cruz, CA, USA).

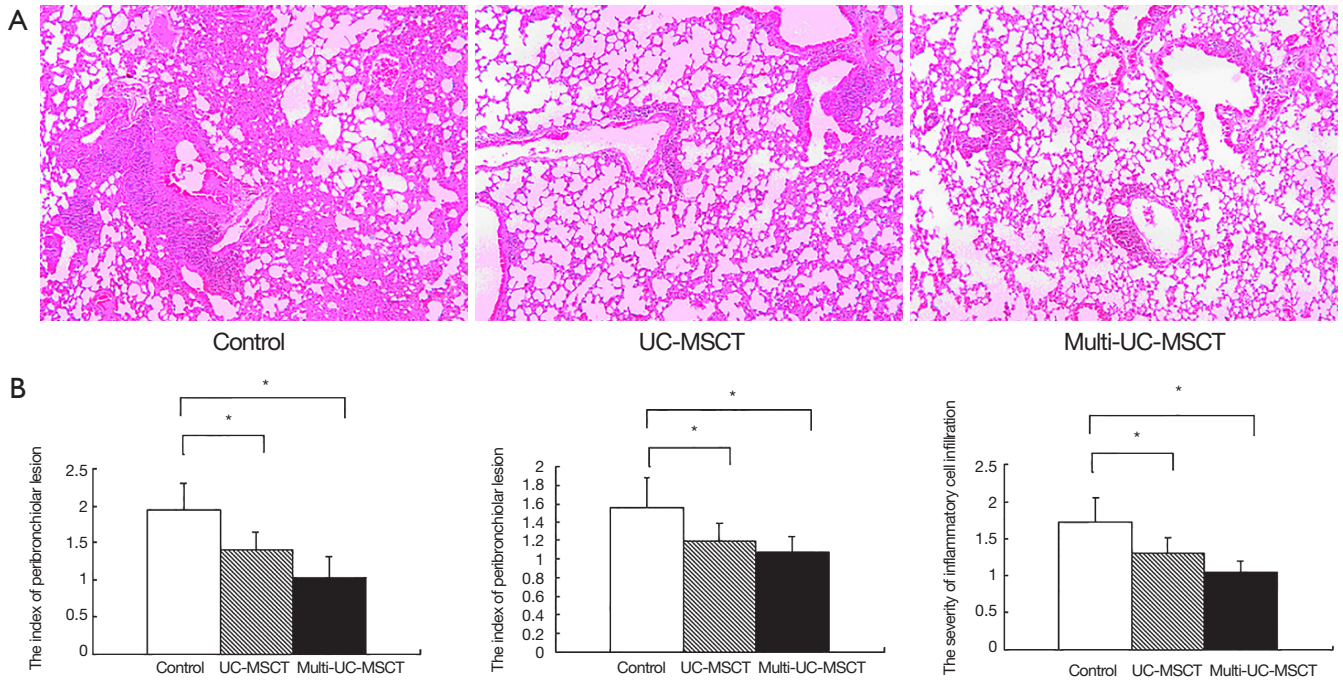

Figure 1 UC-MSCT improved pulmonary pathological injury in MRL/lpr mice. (A) UC-MSCT and multi-UC-MSCT reduced perivascular and peribronchiolar infiltration of inflammatory cell, improving vascular congestion and edema (H&E staining); (B) The degrees of pneumonitis of control and treated MRL/lpr mice were scored as described in Materials and methods. Values are presented as the mean and standard deviation (n=8 mice per group). All treatments (UC-MSCT and multi-UC-MSCT) exhibited a lower index of perivascular and peribronchiolar lesion (*P<0.05 *vs.* control). The severity of alveolar inflammatory cell infiltration was also much slighter (*P<0.05 *vs.* control).

Band detection was conducted using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, USA).

Statistical analysis

Quantitative data were expressed as mean ± 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 pneumonitis of MRL/lpr mice

As mentioned above, there were three groups in the present study: control, UC-MSCT and multi-UC-MSCT. Two mice died respectively at 26 and 28 weeks of age in the control, which were also included in the following analysis. In control group, MRL/lpr mice showed typical interstitial lung disorders according to histopathology such as the perivascular and peribronchiolar focal aggregation of lymphocyte and mononuclear cell (*Figure 1A*). Besides the inflammatory cell infiltration, vascular congestion and edema were found in pulmonary interstitial. We found that all the treatment groups showed much less inflammatory cell infiltration in comparison with control mice. The index of perivascular and peribronchiolar lesion and the severity of inflammatory cell infiltration were much slighter (*Figure 1B*, *Table 1*). Further histological analysis demonstrated that the lungs from the treatment groups showed reduced deposition of collagen (*Figure 2*). In addition, the degree of lung injury in the multi-UC-MSCT group was significantly lower than that in the UC-MSCT. These findings suggest that UC-MSCT is a superior therapeutic approach for treating pneumonitis of MRL/lpr mice. Multi-infusion of UC-MSCs may enhance their effects.

UC-MSCs transplantation decreases the expression of HMGB-1 in lung of MRL/lpr mice

Recent studies have showed that HMGB-1 play an important

Table 1 The effect of UC-MSCT on pulmonary pathological injury in MRL/lpr mice

Group	Number	The index of peribronchiolar lesion	The index of perivascular lesion	The severity of inflammatory cell infiltration
Control	8	1.95±0.35	1.56±0.32	1.72±0.34
UC-MSCT	8	1.40±0.24*	1.20±0.18*	1.30±0.21*
Multi-UC-MSCT	8	1.02±0.29*	1.08±0.16*	1.05±0.15*

*, P<0.05 as compared to control.

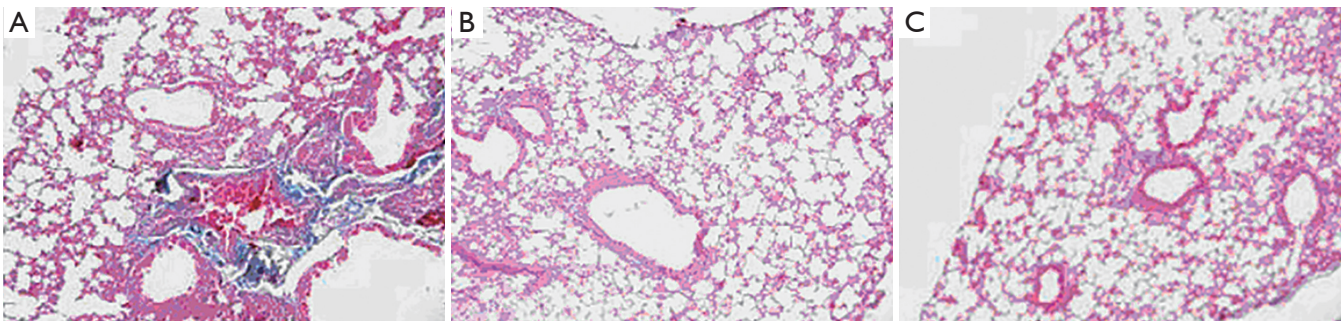

Figure 2 UC-MSCT and multi-UC-MSCT reduced deposition of collagen in the lung of MRL/lpr mice (Masson staining) (A-C).

role in the pathogenesis of SLE (4,22,23). We found that the expression of HMGB-1 protein in all the treatment mice was significantly lower than in control (*Figure 3A*,*B*; P<0.05). The differences in mRNA expression corresponded well with protein expression (*Figure 3C*, *Table 2*). Immunohistochemical staining for HMGB-1 showed marked intense staining in the control lungs. This positive staining was much weaker among all the treatment groups (*Figure 3D*). These results indicate that transplanting UC-MSCs is effective in the treatment of SLE pneumonitis, possibly by inhibiting HMGB-1 expression.

Discussion

SLE is sometimes a severe and thorny disease that often represents a therapeutic challenge because of its heterogeneous organ manifestations. Symptomatic pulmonary manifestations occur in 40% to 50% of the patients with SLE during the course of the disease (24). At autopsy, histological changes associated with SLE are found in almost all cases (25). Pneumonitis in SLE can be a severe and potentially life-threatening complication even despite current optimized therapy. The pulmonary manifestation of lupus is an important indicator of overall prognosis (26). Generally, pneumonitis in SLE patients will primarily be treated with glucocorticoids, cytotoxic and immunoregulatory agents (27). Alveolar haemorrhage is seen as an indication for additional plasma exchange (28-30). Rituximab has also been reported to provide benefit in these conditions (31-33) and infliximab has suggested effective to ILD which is refractory to cyclophosphamide (30). Though, the severe and life-threatening pulmonary manifestations in SLE, such as the acute course of lupus pneumonitis and the more smouldering course of ILD, represent a therapeutic challenge.

Previous studies about MSCs and ILD mainly concentrated in bleomycin (BLM) induced lung injury. MSCs can homing to and locate in the damaged lung tissue (34-36). After transplanting MSCs from male BALB/c rats to female C57BL/6 rats which have been caused lung damage by BLM, Ortiz *et al.* have found that donor derived MSCs can be settled at the site of injury of pulmonary receptors induced by BLM, and showed the epithelial like morphology, and can reduce the degree of inflammation and collagen deposition (35). Studies from Zhao *et al.* showed that MSCs in rat lung tissue damaged of BLM to differentiate into alveolar epithelial cells and bronchial epithelial cells (36). Rojas *et al.* (34) has also found that the protective mechanism of MSCs on lung injury induced by BLM is related to not only the MSCs differentiation to specific phenotype of lung cell, but also the increasing granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage

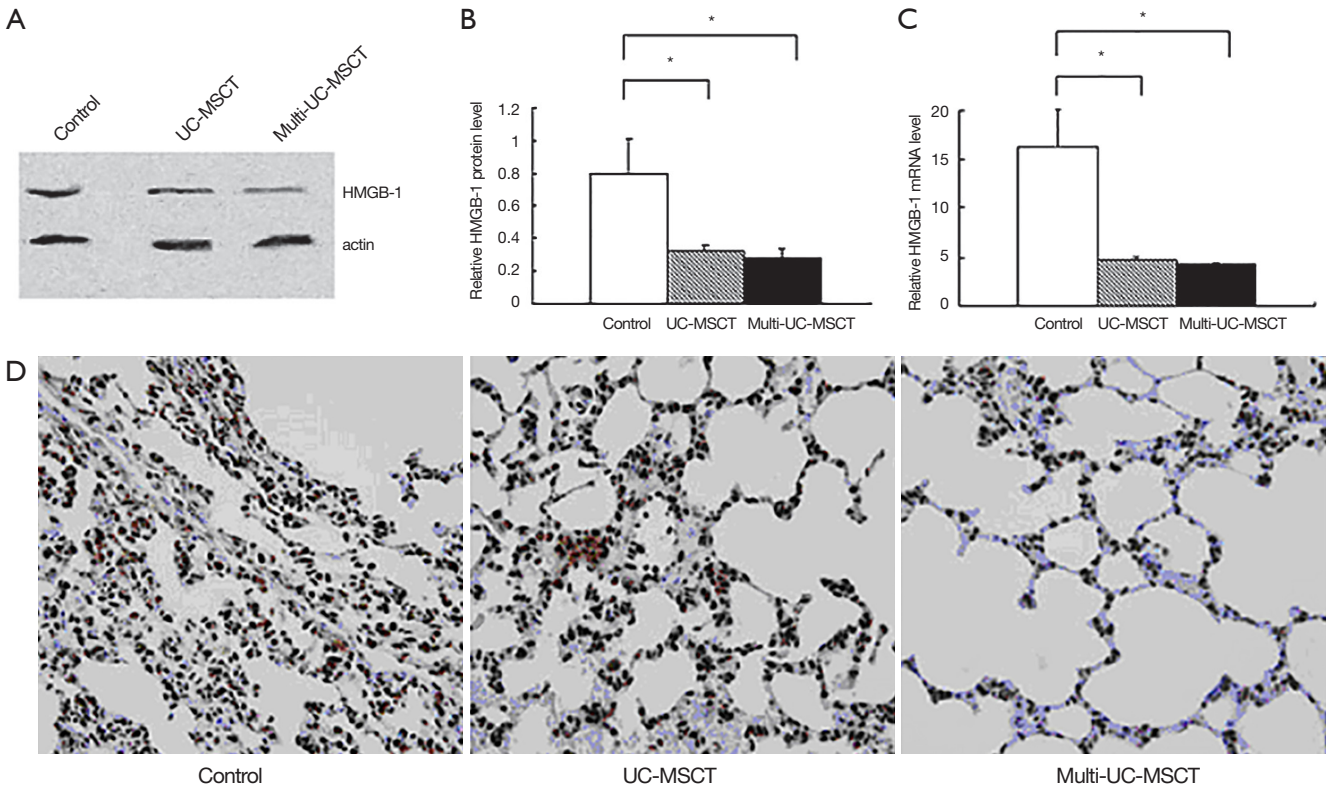

Figure 3 UC-MSCs treatment decreased the expression of HMGB-1. (A,B) HMGB-1 protein expression in control lungs was significantly higher than in all the treatment groups (*P<0.05 *vs.* control). β-actin was used as loading controls in western blot. Three repeated tests per group showed similar results; (C) HMGB-1 mRNA expression in control lungs was significantly higher than in all the treatment groups (*P<0.05 *vs.* control). GAPDH was used as internal control in RT-PCR. Three repeated tests per group showed similar results; (D) The expression of HMGB-1 in lung tissues was represented by immunohistochemical analysis. In the UC-MSCT and multi-UC-MSCT groups, lung HMGB-1 expression was significantly lower than that in the control group. Representative photomicrographs of the lung immunohistochemistry (×400) showed the increased redistribution of HMGB-1 from nucleus to cytoplasm and extracellular areas.

Table 2 Relative HMGB-1 mRNA level in MRL/lpr mice

Group	Number	Relative HMGB-1 mRNA level
Control	8	16.39±3.56
UC-MSCT	8	4.66±0.37*
Multi-UC-MSCT	8	4.45±0.10*

*, P<0.05 as compared to control.

colony stimulating factor (GM-CSF) which promote its own stem cell mobilization, and the decreasing release of inflammation factors involved in.

In the present study, we have demonstrated that transfusions of xenogeneic UC-MSCs significantly attenuate the severity of lung injury in MRL/lpr mice. There were significant differences in the airtube and vascellum injury levels between the treatment and control groups. Light microscopic examination of the lung tissues showed that the improvement of pulmonary pathology correlates well with reduced deposition of collagen and the infiltration of the interstitial inflammatory cell. It is of interest that three transfusions provided more significant reduction in the above-mentioned disease activity manifestations.

HMGB-1, originally characterized as a nuclear DNA-binding protein to be a regulator of transcription, has also been described to have an extracellular role when it is involved in cellular activation and proinflammatory responses (3,37-39). Monocytes and macrophages stimulated by LPS, TNF-α, or IL-1, secrete HMGB-1 (40,41). Addition of HMGB-1 to monocytes, macrophages, or neutrophils in culture induces the release of proinflammatory cytokines, including TNF-α, IL-1α, IL-1β, IFN-γ, MIP-2, and IL-8 (40-43). It can also activate the endothelial cells, increasing the expression of vascular cell adhesion molecule and cell adhesion molecules (44), which leads to the accumulation of inflammatory cells in the vascular wall to produce

vasculitis. HMGB-1 has been considered as a new pattern of inflammatory factor.

HMGB-1 has also been shown to act as an endogenous immune adjuvant 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 (45,46). Interestingly, it was recently shown that HMGB-1 and RAGE mediated TLR9-dependent activation of plasmacytoid dendrite cells by DNA-containing ICs (47). Thus, HMGB-1 plays an important role on the function of the immune system.

SLE is an autoimmune disease, whose pathological basis is vasculitis. Anti-HMGB-1 antibody was found in SLE patients (22). Popovic *et al.* colleagues have found high amounts of extracellular HMGB-1 in skin lesions of lupus (23). Deocharan and his colleagues found that immunization of non-autoimmune mice with a-actinin induced strong anti-nuclear antibody (ANA) response, particularly against chromatin. Furthermore, kidney glomerular IgG deposition and proteinuria were present in a-actinin-immunized mice (48). All above indicated HMGB-1 had an important effect on the genesis and development of SLE.

Al-Mutairi *et al.* reported that proinflammatory cytokines (TNF-α, IFN-γ, IL-8, IL-6) were more prevalent in the serum of SLE patients with pulmonary involvement compared with those without pulmonary manifestations (49). And HMGB-1 is deeply involved in inflammation and immunity. Studies from Maria *et al.* showed that inhibition of HMGB-1 protects against pseudomonas aeruginosa pneumonia in cystic Fibrosis (50). In the present study, we showed that the expression of HMGB-1 in lung 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 pneumonitis by UC-MSCs.

In summary, our study has shown that infusion of UC-MSCs exerts a therapeutic effect in treating pneumonitis in MRL/lpr mice without obvious major side effects. UC-MSCs were able to improved pulmonary pathological injury, reduced inflammatory cell infiltration, and reduced HMGB-1 expression in MRL/lpr mice. The results demonstrate that UC-MSCs could effectively prevent the development of pneumonitis of SLE. However, it remains to be determined whether UC-MSCs transfusions will reverse progression of established pneumonitis in SLE. 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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