LABORATORY STUDY

Human embryonic mesenchymal stem cells alleviate pathologic changes of MRL/Lpr mice by regulating Th7 cell differentiation

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ABSTRACT
Recent evidence indicates that mesenchymal stem cells (MSC) derived from early embryonic tissues have better therapeutic ability as compared with adult tissue-derived stem cells. In the present study, we transplanted human early embryonic MSC (hMSC) into MRL/Lpr mice via tail vein injection to observe the therapeutic efficacy of hMSC and their impact on T helper 17 (Th17) cell differentiation in MRL/Lpr mice. Animals in hMSC treatment group received hMSC (1 × 10^6/200 μL) via the tail vein at the age of 16 and 19 weeks. We found that hMSC treatment prolonged the survival of MRL/Lpr mice without inducing tumorigenesis, reduced urine protein, and alleviated the renal pathologic changes. In addition, it reduced the proportion of Th17 cells in the spleen of MRL/Lpr mice and the serum interleukin 17 (IL-17) concentration. Our in vitro experiment also demonstrated that hMSC could secrete Th17 differentiation-related cytokines of PGE2, IL-10 and TGF-β, and IFN-γ stimulation up-regulated the secretion of these immune regulating factors. The results of the present study suggest that hMSC therapy could alleviate systemic and local renal lesions in MRL/Lpr mice, probably by secreting immune regulating factors and regulating Th17 cell differentiation in MRL/Lpr mice.

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Introduction
Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease affecting multiple organs and systems, the clinical manifestations of which vary greatly from mild rash to severe organ pathologies.1 Lupus nephritis (LN) is a common and severe complication of SLE.2

T helper 17 (Th17) cells are a subset of T cells that can secrete interleukin 17 (IL-17) and play important roles in autoimmune diseases and defense responses of the body. IL-17 is the main effector secreted by Th17 cells, working as a pro-inflammatory cytokine that has a powerful ability to recruit neutrophils to promote various cell types to secrete inflammatory factors. Studies demonstrated that Th17 and IL-17 were closely associated with the development and progression of the disease in both SLE patients and animal models.3–5 Serum IL-17 level was elevated in SLE patients, and this elevation was positively correlated with the degree of SLE activity. Infiltration of Th17 cells was observed in the skin, lung, and kidney of SLE patients.3–5

Several animal6,7 and clinical studies8,9 have demonstrated that mesenchymal stem cell (MSC) therapy can be used as a safe and effective option for the treatment of SLE. MSCs from different tissue sources have different abilities of proliferation and differentiation. Compared with tissue-derived MSC of late-stage differentiation, early tissue-derived MSC have stronger abilities of proliferation and differentiation and therefore have more powerful abilities of repairing the injured organs.10–12 There are no many studies reporting the treatment of SLE with embryo-derived MSC. The aim of the present study was to observe the therapeutic effect of human early embryonic MSC (hMSC) on MRL/Lpr mice and explore the immune mechanism of their injury-repairing ability, knowing that hMSCs have relatively low immunogenicity, low histocompatibility antigen expression, and strong proliferative, differentiative, and immunoregulatory abilities.13
Materials and methods

Cell culture and incubation

hMSCs from human embryos aged 4–7 weeks were provided by Dr. Minjuan Wu (Department of Histology and Embryology of the Second Military Medical University). They were grown as described previously, stored in the Department of Histology and Embryology of the Second Military Medical University (Shanghai, China), and maintained in Dulbecco’s minimal essential medium (DMEM, Invitrogen, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS) at 37°C in an atmosphere of 5% carbon dioxide.

In vivo experiments

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised on 1996). MRL/Lpr mice aged 10 weeks were purchased from the Laboratory Center of Nantong University (Nantong, China) and housed in a constant temperature room with a 12-h dark/12-h light cycle with free access to standard laboratory chow and tap water. 24-h urine was collected weekly by using the metabolic cage for determination of 24-h urine protein. At week 16, mice with 24-h urine protein level greater than 100 mg/dL were included in this study and randomized to two groups: normal saline (NS) group (n = 16) and hMSC group (n = 16). Animals in hMSC group received 300 μL hMSC (1 × 10⁶ cells) via the tail vein at weeks 16 and 19, and animals in NS group received the same amount of NS via the tail vein at the same time points.

Renal function was assessed in terms of serum creatinine (SCR) and blood urea nitrogen (BUN) determined by enzymatic colorimetric assay at week 24. Kidneys were harvested for histological analysis.

Renal morphology

Kidney samples were fixed in 4% neutral buffered formaldehyde for 72 h, sliced into 2-μm sections, and stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). The severity of glomerulonephritis was evaluated in a blinded manner by histological examination of the sectioned kidneys, with results expressed as the crescent formation rate. The glomerular pathology was carried out by assessing 100 glomerular cross sections (GCS) per kidney, and the crescent formation rate was defined as the number of crescent-formed glomeruli/GCS. Interstitial inflammatory cell accumulation was determined semi-quantitatively by scoring the number mononuclear cells into interstitial spaces using the scores of mild (1, <25%), moderate (2, 25–50%), or extensive (3, >50%).

Detection of Th17 cells in the mouse spleen by flow cytometry

The spleen was removed by aseptic technique and ground in mouse lymphocyte separation medium to collect the filtrate using a nylon sieve, with the fluid surface covered by addition of 500 μL RPMI1640 medium (Gibco, Grand Island, NY). The filtrate was then centrifuged at 2500 rpm for 20 min until it was separated in four layers. The layer containing mononuclear cells was sucked to the centrifuge tube containing 3 mL Hank’s solution, washed three times and counted. The mouse spleen mononuclear cells were prepared as a 200 μL cell suspension and placed in a 24-well plate, added with PMA + ionomycin and BFA + monensin in each well, and cultured in a 5% CO₂ incubator at 37°C for 4–6 h. The post-stimulation cultured cell suspension was transferred to the corresponding flow test tube, added with CD4-FITC (eBioscience, Cat. 12-7177), incubated at room temperature away from light for 30 min, added again with 100 μL Fix&Perm, incubated at room temperature away from light for 15 min, and washed once. After discarding the supernatant, each tube was added with 100 μL Fix&Perm containing the membrane-breaking agent and hemolysate, together with PE-IL-17A (eBioscience, Cat. 12-7177), incubated at room temperature away from light for 30 min, and washed once to discard the supernatant. Cells were re-suspended in phosphate-buffered saline and detected on the machine. The T lymphocyte subset was chosen on the SSC scatter diagram. Using CD4/SSC gating method, CD4⁺ cells were chosen to analyze the expression of IL-17. The number of cells collected was 10,000 cells/tube.

Detection of serum IL-7 and dsDNA, cell culture fluid, IL-10, PGE2, and TGF-β secretion by ELISA

Anti-dsDNA antibody, IL-7, IL-10, PGE2, and TGF-β in mouse frozen serum and cell culture fluid were detected according to the instructions of the ELISA kits (R&D Systems, Minneapolis, MN). Knowing that IFN-γ plays a key role in the development and progression of SLE, the mean IFN-γ level is elevated in SLE patients and animal models and this elevation is positively correlated with the degree of SLE activity, we used 200 U/mL IFN-γ (R&D Systems, Minneapolis, MN) to pre-treat hMSC for 24 h to simulate the SLE inflammatory environment within the body and detect the expression level of PGE2, IL-10, and TGF-β before and after stimulation. The supernatant was harvested from the control and
pretreatment groups after 24-h treatment, centrifuged to remove the deposit, and stored at −20 °C for analysis.

Statistical analysis
Data were analyzed by standard statistical methods and analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons or the nonparametric Kruskal–Wallis test. Group data are expressed as means ± SD, and \( p < 0.05 \) was considered statistically significant. Kaplan–Meier statistics were used for survival analysis.

Results
hMSC therapy prolongs the survival and reduces anti-dsDNA antibodies in MRL/Lpr mice
The hMSC therapy prolonged the length of survival of the SLE mice markedly. The death rate in hMSC group was significantly lower than that in NS group. The survival rate in hMSC group was twice as high as that in NS group by 24 weeks after treatment (87.5% vs. 43.75%, Figure 1(A)). Compared with the NS group mice, the hMSC group mice were more active, and their cutaneous injury and epilation were alleviated significantly. Weight increase with age in NS group was slower than that in hMSC group. From 20 weeks of treatment, the mean body weight in hMSC group was significantly higher than that in NS group \( (p < 0.05, \text{Figure 1(B)}) \). At week 24, the serum dsDNA level in hMSC group was significantly lower than that in the NS group \( (425.71 ± 57.02 \text{ vs. } 492.55 ± 37.89, p < 0.05, \text{Figure 1(C)}) \).

Effect of hMSC therapy on the pathologic changes of the kidney in MRL/Lpr mice
At 18 weeks, the urine protein level in hMSC group was significantly lower than that in NS group, and at 24 weeks it was \( 2.53 ± 1.04 \) in hMSC group vs. \( 8.52 ± 4.98 \) in NS group, showing a 70% decrease between the two groups \( (p < 0.01, \text{Figure 1(A)}) \). The urine protein level in the NS group increased significantly with time prolonging \( (\uparrow p < 0.05 \text{ vs. 18, 20, 22, 24 weeks, Figure 1(A)}) \), and at 24 weeks it increased about 4-fold as compared with that at 16 weeks, while in hMSC group the urine protein level increased insignificantly at all time points except at 20 weeks when it increased slightly as compared with that at 16 weeks \( (\uparrow p < 0.05 \text{ vs. 16 weeks in hMSC, Figure 2(A)}) \). The serum protein level in hMSC group was significantly higher than that in NS group at 24 weeks \( (*p < 0.05 \text{ vs. NS, Figure 2(B)}) \), and

Figure 1. hMSC treatment prolongs survival and reduced levels of anti-dsDNA antibodies in MRL/Lpr mice. (A) hMSC treatment prolongs survival \( (p < 0.01 \text{ vs. NS}) \). (B) hMSC treatment increased the body weight \( (*p < 0.05 \text{ vs. NS}) \). (C) There was a significant difference between the levels of anti-dsDNA antibody in hMSC and that in NS \( (*p < 0.05 \text{ vs. NS}) \).
there was no significant difference in serum BUN and Scr between the two groups (p > 0.05, Figure 2(C and D)).

**Histopathologic findings**

PAS and H&E staining was performed on all kidney tissues of the two groups. At week 24, proliferation of glomerular mesenteric cells and mesenteric matrix, shrinkage of capillary loops, crescent formation in partial glomerular cells, and periglomerular infiltration of inflammatory cells were observed in NS group. Compared with NS group, proliferation of glomerular mesenteric cells and mesenteric matrix was alleviated, crescent formation was reduced (0.27 ± 0.07 vs. 0.06 ± 0.06, p < 0.05, Figure 3(A and B)), interstitial infiltration of inflammatory cells was alleviated significantly (3.00 ± 0.89 vs. 1.17 ± 0.75, p < 0.05, Figure 3(A and C)).

**hMSC therapy reduces the proportion of Th17 cells in the spleen of MRL/Lpr mice and serum IL-17 level**

Compared with NS group, both the Th17/lymphocyte ratio in the spleen of hMSC group mice and serum IL-17 level were decreased significantly (2.56 ± 0.22 vs. 1.08 ± 0.27, p < 0.05, Figure 4(A and B); 100.34 ± 17.25 vs. 83.19 ± 8.41, p < 0.05, Figure 4(C)).

**INF- stimulation up-regulates hMSC and promotes IL-10, PGE2, and TGF-β secretion**

hMSC could secrete IL-10, PGE2, and TGF-β and this secretion ability was enhanced after 24-h INF-γ stimulation (p < 0.05, Figure 5(A)), especially the secretion of PGE2 and TGF-β, which increased by about 10-fold (17,200 ± 5630 vs. 1444 ± 382, p < 0.01) and 3-fold (916 ± 202 vs. 238 ± 67.6, p < 0.01, Figure 5(B and C)), respectively, as compared with those before stimulation.

**Discussion**

MSCs can be separated from embryos and adult tissues. Compared with adult tissue-derived MSC, fetus-derived MSC have greater amplification and differentiation potentials. Immunologically, histocompatibility antigens are less intensively expressed in embryo-derived cells of early gestation terms than those in adult cells. There are not many studies reporting the treatment of SLE with embryo-derived MSC. The hMSC used in this study were isolated from 4- to 6-week-old
embryos from volunteers who underwent pregnancy termination with RU-486, an antiprogesterone compound.\textsuperscript{13} Our previous studies\textsuperscript{17,18} suggested that hMSC possessed the potential to differentiate into dermal papilla cells, and EGF-transfected hMSC could differentiate to epithelial-like cells under Ca\textsuperscript{2+} stimulation. Prior studies\textsuperscript{19,20} also found that hMSC had a protective effect on organs sustained cisplatin-induced acute kidney injury and bleomycin-induced lung injury in mice.

Several studies\textsuperscript{6–9} have demonstrated that MSC therapy could achieve remarkable therapeutic outcomes in SLE patients and animal models. Zhou et al.\textsuperscript{6} used human bone marrow MSC (BMSC) and Gu et al.\textsuperscript{7} used mouse-derived MSC to treat SLE in MRL/Lpr mouse models for 33 weeks and found that the survival rate in the treatment groups was 100\% versus 40\% and 55\% in the control groups. Consistent with these previous studies, we also found that hMSC had a good therapeutic effect in MRL/Lpr mice, as represented by the prolonging of survival time, decrease in urine protein and alleviation of the renal pathologic changes. The present study showed that the survival rate in hMSC group and NS group was 87.5\% and 43.75\% respectively at 24 weeks, but there was no significant difference in renal function change between the two groups,

![Figure 3](image-url)

Figure 3. hMSC transplantation alleviates lupus nephritis in MRL/Lpr mice. (A) Representative hematoxylin and eosin staining of renal sections from each group. Original magnification 400×. (B) The degree of crescent formation and (C) the tubulointerstitial inflammatory cell infiltration score in hMSC group was significantly lower than in the control group (*p < 0.05 vs. NS).
probably because the systemic pathology of the MLR/Lpr mice in our study was relatively severe and the duration of observation was relatively short.

The imbalance between Th1/Th2 and Treg/Th17 ratios plays an important role in the development and progression of SLE.²¹ It was reported²² that IL-12, 21, and 23 increased with increased proliferation of Th17 cells in the body of SLE patients, and these changes may cause the imbalance in Th17/Th1 ratio and Treg cell depletion. It was found in both SLE patients and animal models that IL-17 worked together with IL-21 and BAFF on the germinal center, thus affecting B cells to generate various antibodies against immune diseases.²³,²⁴ The expression level of glomerular IL-17 in LN patients was positively correlated with the renal activity score and histopathology of SLE patients.²⁵ Therefore, Th17 may prove to be a new therapeutic target for the treatment of SLE, probably by blocking the differentiation and proliferation of Th17 cells, inhibiting or neutralizing cytokines secreted by Th17 cells, and inhibiting Th17 cell-specific transcription factors.²⁶ In the autoimmune environment, MSC can improve the dysfunction of the T-cell subset.²⁷ Studies concerning MSC treatment of SLE patients²⁸ and lupus animal models²⁹,³⁰ showed that either autogenic, xenogenic, or umbilical BMSC could inhibit Th17 differentiation by increasing the number of Treg cells, which is consistent with the finding of the present study that hMSC therapy could down-regulate the Th17 cell ratio in the spleen of MLR/Lpr mice and the serum IL17 concentration, suggesting that the therapeutic efficacy of hMSC is associated with their inhibitory effect on Th17 cell differentiation and decreased secretion of IL-17.

The mechanism of SLE treatment with MSC involves multiple aspects, including direct differentiation of MSC into healthy tissues and organs, immunomodulation, and paracrine and endocrine effects, of which immunomodulation is an important factor, though the exact mechanism and outcome remain indefinite. Some studies suggested that it may involve multiple pathways.³⁵ Many factors are involved in MSC immunomodulation, including but not limited to, the production of IL10, PGE2, TGF-β, and other soluble factors.³⁶ These factors directly or indirectly affect the differentiation of Th17 cells in varying degrees.

Studies on myocardial infarction and inflammatory bowel diseases³¹ showed that IL-10 could directly or indirectly through Treg inhibit the differentiation of Th17 cells. Qu et al.³² found that either neutralization of IL-10 secreted by mouse BMSC or down-regulation of IL-10 in BMSC using the RNA interfering technique could attenuate the inhibitory effect of BMSC on Th17 cell differentiation. In a mouse model of experimental autoimmune encephalomyelitis, Payne et al.³³ found that compared

Figure 4. hMSC transplantation inhibited Th17 cell differentiation and decrease serum level of IL-17. (A) Flow cytometric analysis showed percentage of Th17. (B) hMSC treatment inhibited Th17 cell differentiation and (C) decrease serum level of IL-17 (*p < 0.05 vs. NS).
with GFP-MSC, IL-10 modified MSC could achieve better therapeutic efficacy by directly acting on pathologic T cells and inhibiting Th17-mediated immune response through suppressing maturation of the dendritic cell phenotype, generation of cytokines, and the antigen-presenting function.

There are discrepancies over the immunoregulatory effect of PGE2 on Th17 differentiation. Some studies believed that PGE2 exerted its anti-inflammatory effect by inhibiting Th17 differentiation, while others provided evidence that PGE2 promoted Th17 differentiation.\textsuperscript{34,35} However, either \textit{in vivo} or \textit{in vitro} studies suggested that MSC inhibited Th17 differentiation by secreting PGE2. Bouffi et al.\textsuperscript{36} found that PGE2 secretion from MSC depended on IL-6 and MSC therapy was effective for the treatment of collagen-induced arthritis in a mouse arthritis model, but MSC deprived of IL-6 could not relieve arthritis. These findings suggest that the therapeutic effect of MSC is related to the inhibition of Th7 differentiation by PGE2 secreted from MSC.

Serum TGF-\(\beta\) level in SLE patients is lower than that in normal populations. There may exist TGF-\(\beta\) resistance in SLE patients and therefore decreased TGF-\(\beta\) may be one of the mechanisms of organ injury in SLE patients.\textsuperscript{37} It was reported\textsuperscript{38} that TGF-\(\beta\) up-regulated the expression of orphan nuclear receptor (ROR-\(\gamma\)t), induced the generation of Treg cells, inhibited Th17 cell differentiation, and finally reduced IL-17 secretion. Kong et al.\textsuperscript{39} found that TGF-\(\beta\) secreted from BMSC could partially correct the imbalance of the Th1/Th2 and Th17/Treg ratios.

Compared with adult tissue-derived MSCs, fetal stem cells, and embryo-derived stem cells have a powerful immunoregulatory effect, and this difference is closely associated with the ability and type of cytokines that they secrete.\textsuperscript{10,40} Although TGF-\(\beta\) and PGE2 secretion can be observed in various types of MSC, the results of studies about IL-10 obtained from different species and MSC sources are not consistent. There are studies reported that both human fetus- and placenta-derived MSC could secrete IL-10\textsuperscript{41}; however, MSC from the human body could not secrete IL-10.\textsuperscript{40,42} Fetus-derived MSC had a better immunoregulatory effect than adult tissue-derived MSC, and the immunoregulatory effect of fetus-derived MSC was associated with the cytokines secreted, including PGE2, IL-10, and TGF-\(\beta\).\textsuperscript{43,44} In the present study, we used MSC from early embryos, similar to the above-mentioned MSC from early sources, and detected IL-10, PGE2, and TGF-\(\beta\) in the supernatant of hMSC, suggesting that these cytokines may participate in the regulation of hMSC on Th17 differentiation.

IFN-\(\gamma\) plays a key role in the development and progression of SLE. It was found\textsuperscript{15} that the IFN-\(\gamma\) level was elevated in both SLE patients and animal models, and this elevation was positively correlated with the degree of SLE activity. In the present study, we stimulated hMSC by using IFN-\(\gamma\) to simulate the inflammatory environment in the human body and detected the expression of PGE2, IL-10, and TGF-\(\beta\) before and after stimulation. It was found that IFN-\(\gamma\) could up-regulate hMSC to secrete the above immune-regulatory factors.
Although fetal stem cells have been shown capable of proliferation and differentiation, tumorigenesis limits their further clinical application. hMSCs used in this study were derived from embryos aged 4–7 weeks. No tumorigenesis was observed in previous studies using hMSC and vascular endothelial growth factor-modified hMSC to treat acute kidney injury in nude mice.19 The safety of using hMSC in the treatment of MRL/Lpr mice was also confirmed in our study, knowing that no tumorigenesis was observed three months after hMSC grafting.

We have demonstrated that hMSC could prolong the survival of MRL/Lpr mice without inducing tumorigenesis, reduce urine protein and anti-dsDNA antibodies, and alleviate renal pathologic changes. hMSC could secrete PGE2, IL-10, and TGF-β, and IFN-γ could upregulate hMSC to secrete these immune regulatory factors. Both the proportion of Th17 cells in the mouse spleen and the serum IL-17 concentration were decreased in MRL/Lpr mice after hMSC treatment, suggesting that hMSC exert their therapeutic effect on MRL/Lpr mice probably by inhibiting immune-regulatory factors and regulating Th17 cell differentiation. These findings may provide a new promising option for stem cell treatment of SLE.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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