Long-Term Therapeutic Effects of Mesenchymal Stem Cells Compared to Dexamethasone on Recurrent Experimental Autoimmune Uveitis of Rats

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PURPOSE. We tested the long-term effects of different regimens of mesenchymal stem cell (MSC) administration in a recurrent experimental autoimmune uveitis (rEAU) model in rats, and compared the efficacy of MSC to that of dexamethasone (DEX).

METHODS. One or two courses of MSC treatments were applied to R16-specific T cell-induced rEAU rats before or after disease onsets. The DEX injections were given for 7 or 50 days continuously after disease onsets. Clinical appearances were observed until the 50th day after transfer. On the 10th day, T cells from control and MSC groups were analyzed by flow cytometry. Supernatants from the proliferation assay and aqueous humor were collected for cytokine detection. Functions of T cells and APCs in spleens also were studied by lymphocyte proliferation assays.

RESULTS. One course of MSC therapy, administered after disease onset, led to a lasting therapeutic effect, with a decreased incidence, reduced mean clinical score, and reduced retinal impairment after 50 days of observation, while multiple courses of treatment did not improve the therapeutic benefit. Although DEX and MSCs equally reduced the severity of the first episode of rEAU, the effect of DEX was shorter lasting, and DEX therapy failed to control the disease even with long periods of treatment. The MSCs significantly decreased T helper 1 (Th1) and Th17 responses, suppressed the function of antigen-presenting cells, and upregulated T regulatory cells.

CONCLUSIONS. These results suggested that MSCs might be new corticosteroid sparing agents, while providing fewer side effects and longer lasting suppressive effects for recurrent uveitis.

Keywords: mesenchymal stem cells, recurrent experimental autoimmune uveitis, dexamethasone, T cells, antigen-presenting cells

Autoimmune uveitis, especially chronic and recurrent uveitis involving the choroid and retina, frequently results in numerous severe clinical complications, including cataract, glaucoma, cystoid macular edema, and retinal damage, and is a major cause of visual disability, worldwide. New therapies, especially biologics, have been developed successfully to decrease the corticosteroid burden on patients, while managing refractive uveitis.^{1,2} However, serious potential side effects, including reactivation of latent tuberculosis and malignant diseases, may limit their use, and a significant percentage of patients still do not respond well to these treatments, indicating that targeting one specific mediator is not effective in all cases. Therefore, development of effective therapeutic approaches still is an important and urgent clinical objective.

Mesenchymal stem cells (MSCs) can inhibit the autoimmune response, allograft rejection, and graft-versus-host disease

(GVHD), by targeting different parts of the immune system.³ The MSCs are superior to corticosteroids and immunosuppressants in respect that they also possess neurotrophic and antimicrobial effects, and, therefore, are considered safe regarding risk of infection.^{4–6} Although their therapeutic effects have been exploited successfully in a number of experimental immune-mediated diseases, and recently are being evaluated with encouraging early results in a range of clinical conditions,^{7–11} further data are necessary to optimize the regimens and establish clear guidelines for the administration of MSC-based therapies.

Experimental autoimmune uveitis (EAU) closely resembles the immunopathologic features of human posterior uveitis, and can be induced in susceptible animals by immunization with retinal proteins, or transfer of antigen specific T cells or dendritic cells (DCs).¹² Among various established EAU models,

recurrent EAU (rEAU), induced by injection of photoreceptor retinoid binding protein (IRBP)-specific T cells into rats, exhibits resolution of the acute episode with spontaneous recurrences and evidence of chronic, subclinical, intraocular inflammation, and progressive deterioration of retinal structures. Compared to monophasic EAU (mEAU), with spontaneous recovery, this model mimics the situation observed in the human disease and, therefore, provides a useful tool for understanding and developing new therapies for sight-threatening uveitis.¹³

We previously reported that MSCs could ameliorate mEAU in rats by down-regulating T helper 1 (Th1) and Th17 responses, and restoring the balance of Th17/regulatory T cells (Tregs).^{14,15} In this study, we assessed the therapeutic effects and proper regimen of MSC administration, using the rat rEAU model, and compared its therapeutic efficiency to that of dexamethasone (DEX). Compared to DEX, a single course of MSC treatment, administered at the onset of the disease, sufficed to diminish ocular inflammation and reduce the frequency of recurrence, suggesting that MSCs could be an alternative and better treatment for recurrent and chronic uveitis. The beneficial effects of MSCs in rEAU were attributed to a significant decrease in Th1/Th17-mediated inflammation, to regulation of the balance between Th17 and Tregs, and to suppression of the function of antigen-presenting cells (APCs).

MATERIALS AND METHODS

Animals

Male Lewis rats (6–8 weeks) and Wistar rats (4–6 weeks), were purchased from Vital River (Beijing, China), and were housed under pathogen-free conditions. All procedures involving rats were approved by the Laboratory Animal Care and Use Committee of Tianjin Medical University, and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation and Characterization of MSCs

Bone marrow MSCs were isolated from Wistar rats. Briefly, tibiae and femurs were removed aseptically, and the bone marrow was flushed and suspended in PBS. Collected bone marrow cells were incubated in complete media containing Dulbecco's modified Eagle's medium/Nutrient Mixture F12 (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C and 5% CO₂, in a humidified incubator, for at least 24 hours. Nonadherent cells were discarded, and the remaining adherent cells were incubated for 8 to 12 days until they reached approximately 80% confluency. The MSCs on the third to fifth passage were collected for subsequent experiments.

Mature MSCs were defined by their capacity to differentiate into adipocytes, endothelial cells, and osteocytes when cultured under appropriate in vitro conditions. Further characterization was based on the expression of CD90 and CD29, and the absence of cell surface hematopoietic markers, CD34 and CD45, as described previously.¹⁶

Animal Model of rEAU

The induction of rEAU in Lewis rats was performed as described previously.¹³ The EAU was first induced in Lewis rats by active subcutaneous immunization with 200 µg of an emulsion containing 50 µg IRBP peptide R16 (residues, 1177–1191, ADGSSWEGVGVDPV; Sangon, Shanghai, China) and

500 µg *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI, USA) in incomplete Freund's adjuvant (Sigma-Aldrich Corp., St. Louis, MO, USA), distributed over six spots on the tail base and flank. A single cell suspension was prepared on day 10 after immunization, from lymph nodes and spleens of EAU rats, and was added to nylon wool columns. Nonadherent cells were collected as T cells, while the adherent cells were removed from columns after incubation on ice, and irradiated with 30 Gy, to serve as APCs. The T cells (1×10^7 cells/well) then were incubated with APCs (1×10^7 cells/well), at 37°C and 5% CO₂ for 48 hours with stimulation by 10 µg/mL of R16. The T cells then were separated using the Ficoll method. Blasted T cells identified as activated R16-specific T cells were counted under the microscope and accounted as approximately 30% to 50% of the live T cells. For induction of recurrent uveitis, 1×10^7 live T cells were injected intravenously into one naïve Lewis rat.

Treatment With MSCs and DEX

To investigate the preventive, therapeutic, and double course effects of MSCs on rEAU, the rats were treated intravenously with 5×10^6 MSCs, diluted in 1 mL PBS for three consecutive days, starting on day 0 (preventive group), day 4 (therapeutic group), or on days 4 and 15 (double course group). This MSC therapeutic protocol (intravenously injection of 5×10^6 MSCs for three consecutive days) was based on our previous study,^{14,15} which had been proved to be most effective for EAU in rat. To compare the therapeutic effects of MSCs versus DEX, different periods of DEX therapies were used to treat the vehicle-treated rats. The 200 µg/kg DEX was injected intraperitoneally, for 7 successive days. The DEX treatment was discontinued thereafter in the short course group, or continued until the 50th day, with 50 µg/kg reduction, every 10 days, in the long course group.

Clinical and Histological Assessment of rEAU

The rats were examined daily by slit-lamp biomicroscopy, for clinical signs of uveitis. The incidence and severity of inflammation were scored in a masked fashion, on a scale of 0 to 4, according to the criteria of Caspi.¹⁷ Rats were followed up for 50 days after transfer.

Inflammation in the eye and retinal damage were confirmed by histological examination. On day 50 after transfer, eyes were collected and immersed for 1 hour in 4% glutaraldehyde/PBS, and transferred to 10% glutaraldehyde/PBS for at least overnight until further processing. Fixed and dehydrated tissues were embedded in paraffin wax, 4-mm sections were cut through the papillary optic nerve plane, and sections were stained with hematoxylin and eosin. The degree of retinal damage was assessed by measuring the thickness of the retina and outer nuclear layer (ONL). Photographs were taken of the superior and inferior hemispheres, at eight defined points, with a camera attached to a light microscope (Olympus BX51; Olympus, Tokyo, Japan). The first photograph was taken at approximately 1 mm from the center of the optic nerve, and subsequent photographs were taken in a peripheral manner, every 1 mm. The thickness of the whole retina and ONL were measured from the photographs, using CellSen software (Olympus).

Flow Cytometry

On day 10 after transfer, cell suspensions were prepared from eyes, spleens, and lymph nodes. For staining of Foxp3, aliquots of 2×10^5 cells were incubated for 30 minutes at 4°C with anti-

FIGURE 1. Clinical scores in rEAU rats receiving different therapeutic regimen of MSCs. (A) The MSC treatment applied simultaneously with transfer attenuated the inflammation early in the peak phase. (B) The MSC treatment at the disease onset, reduced the severity of inflammation in the peak and recovery phases. (C, D) The MSC treatments significantly reduced the mean clinical scores and incidences during 50 days after transfer, and no differences were found in the preventive, therapeutic, and double therapy groups (twice MSCs). Mean clinical score curves (E1, F1, G1, H1) and typical single score curves (E2, F2, G2, H2) of MSC groups were recorded. Values are expressed as mean $\pm$ SD for 5 rats (10 eyes) per group. * $P < 0.05$. D0–2, therapies were administrated from days 0 to 2; D4–6, therapies were administrated from days 4 to 6.

CD4 (BioLegend, San Diego, CA, USA), then fixed and permeabilized overnight with fixation/permeabilization buffer (Foxp3 staining buffer; eBioscience, San Diego, CA, USA), followed by incubation for 30 minutes at 4°C with anti-Foxp3 (BioLegend) antibody, and washing.

For intracellular cytokine staining, cells were pretreated for 4 to 6 hours with 50 ng/mL phorbol 12-myristate 13-acetate, 1 μ g/mL ionomycin, and 1 μ g/mL brefeldin A (Sigma-Aldrich Corp.), then incubated with anti-CD4 antibody, fixed, and permeabilized overnight, then intracellularly stained with antibodies IFN- γ and IL-17 (BioLegend).

Data collection was performed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), and analyzed using flow cytometry software (FlowJo, Ashland, OR, USA). The acquisition and analysis gates were restricted to the lymphocyte gate, based on characteristic properties of the cells in forward and side scatter.

T Cell Proliferation Assay

T cells and APCs from spleens of either the control group or therapeutic group were separated on day 10 after transfer as described above. T cells (4×10^5 cells/well) were seeded in 96-

well flat bottomed microtiter plates (Corning, Corning, NY, USA) with graded doses of R16 (0, 0.3, 1, and 10 μ g/mL), in the presence of APCs (4×10^4 cell/well), in a total volume of 200 μ L. For crossover experiments, T cells from control groups were cultured in the presence of APCs from MSC-treated groups, and vice versa.

Cells were cultured for 64 hours at 37°C and 5% CO₂, in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin (Invitrogen), and 50 μ M β -mercaptoethanol (ICN Biomedicals, Irvine, CA, USA). The [³H]-thymidine incorporation, after 16 hours of incubation, was assessed by liquid scintillation spectrometry (Accu-FLEXLSC-7200; Hitachi-Alloka Medical Ltd., Tokyo, Japan). The proliferative response was expressed as stimulation index, by dividing the mean counts per minute (cpm) of R16-stimulated cells by the mean cpm of nonstimulated cells.

Cytokine Production

Supernatants from the proliferation assay after 48 hours incubation, and aqueous humor (AqH) on day 10 after transfer, were collected for cytokine detection. The concentrations of IL-17, IL-2, IFN- γ , TNF- α , and IL-10 in conditioned medium and

FIGURE 2. Histological changes of the retina in MSC-treated rEAU rats on day 50. Thicknesses of the retina and ONL were measured at 8 retinal locations at 1-mm intervals (**B**, **D**). (**A**, **C**) Average thicknesses of the retina and ONL. Thicknesses of the retina and ONL of rEAU rats were reduced significantly compared to those of normal rats. The MSC treatments reduced the attenuation of the retina and ONL. There were no statistical differences among the MSC preventive, therapeutic, and double therapy groups regarding thickness of retina, but the MSC therapeutic group showed reduced photoreceptor loss compared to the other two groups. (**E**–**I**) Representative retinal histological features are shown. Values are expressed as mean $\pm$ SD for 5 rats (10 eyes) per group. * $P < 0.05$.

FIGURE 3. Clinical evaluation of MSC and DEX therapies. (A) Clinical score of PBS and DEX administered from days 4 to 10. The DEX significantly reduced ocular inflammation. Inflammation recurred on D12, 2 days after DEX withdrawal. (B) Overlap of clinical score treated with either MSCs or DEX. No significant difference was seen between MSC and DEX therapeutic groups until D12, 2 days after DEX withdrawal. (C, D) The DEX therapy did not alleviate the long-term clinical scores and incidences, and short-term DEX therapy even increased the incidences. Mean clinical score curves (E1, F1) and typical single score curves (E2, F2) of MSC and DEX groups were recorded. Solid arrows indicate relapse of intraocular inflammation and dashed arrows the days of DEX withdrawal and tapping. Values are expressed as mean $\pm$ SD for 5 rats (10 eyes) per group. * $P < 0.05$. D4-10, therapies were administrated from days 4 to 10; D4-50, tapering therapies were administrated from day 4 to 50.

AqH were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's protocols.

Statistical Analysis

We used SAS version 9.2 software (SAS, Cary, NC, USA) for statistical analyses. Data were expressed as means $\pm$ SD. The EAU clinical scores were assessed by repeated measures ANOVA, using mixed models. Student's *t*-test was used for two sets of data, and 1-way ANOVA was used for three or more sets of data. Statistical significance was set at $P < 0.05$.

RESULTS

R16-Specific T Cell Transfer Induced Recurrent EAU With Severe Tissue Damage

Although the courses of rEAU were heterogeneous, the first acute attacks almost always developed with regularity. The clinical symptoms started at 3 to 4 days after transfer, including dilated blood vessels in the iris, abnormal pupil contraction, or a hazy anterior chamber. The disease peaked 2 days later, and was characterized by an opaque anterior chamber, and a dull red reflex or red reflex absent appearance, which then was gradually resolved in 4 to 9 days. The disease relapsed 4 to 6 days later, with less severity compared to the first attack, and the rats experienced 4 to 5 episodes of disease, with gradually decreasing clinical scores, over a period of 50 days. The pattern of clinical symptoms varied from case to case and became moderate or mild at the later stages of the disease. Pathological examination revealed that severe retina damage,

especially photoreceptor loss, developed after multiple clinical relapses.

Single Course of MSC Treatment, Started at the Onset of the Disease, Sufficed to Inhibit the Progress and Relapse of the Disease in rEAU

To investigate the proper time of MSC administration for the rEAU model, MSC transfusions were given simultaneously at the time of transfer (preventive administration), or at the onset of the disease (therapeutic administration), for 3 successive days. In addition, a twice-MSC treatment group was designed to analyze the efficiency of repeated MSC therapy. The rats were given two courses of MSC transfusion in the same manner, at the onset of the disease and at days before the second attack. To evaluate the duration of the effect on the chronic stage of rEAU, we observed the inflammation until 50 days after transfer. Because neither the duration of a single disease peak nor the interval between episodes was predictable in rEAU, we chose the incidence and mean clinical score to evaluate therapeutic effects.

In the first attack, preventive treatment attenuated the disease early in the peak phase (Fig. 1A), while therapeutic treatment reduced inflammation in the peak and recovery phases (Fig. 1B). In the course of 50 days, all MSC regimens significantly reduced the severity and incidences of rEAU. There were no differences between the preventive, therapeutic, and double therapy groups (Figs. 1C-H). Histological examination of the retina showed that MSC treatments significantly reduced retinal damage and photoreceptor loss, while therapeutic MSC administration provided more benefits to the ONL than preventive administration. Regarding retinal protection, consistent with clinical observations no significant

FIGURE 4. Comparisons of histological changes of the retina in MSC- and DEX-treated rEAU rats on day 50. Thicknesses of the retina and ONL were measured at 8 retinal locations at 1-mm intervals (**B, D**). (**A, C**) Average thicknesses of the retina and ONL. Compared to control group, short-term DEX treatment aggravated the damages, and tapering treatment with DEX showed no difference. The MSC therapy in D4-6 showed better results than DEX therapies in protecting the retina and ONL. (**E, F**) Representative retinal histological features are shown. Values are expressed as mean $\pm$ SD for 5 rats (10 eyes) per group. * $P < 0.05$.

advantages of double therapies were observed when compared to the other two MSC groups (Fig. 2).

A Single Course of MSC Treatment Resulted in Better Therapeutic Effects on rEAU Compared to Tapering DEX Therapy

To compare the effect of MSC and traditional corticosteroid therapy for uveitis, rEAU rats were given DEX administration for a short or long course. In the first attack, MSC and short-term DEX therapy efficiently protected against ocular inflam-

mation (Figs. 3A, 3B). There was no statistical difference between MSC and DEX treatments. With a longer period of observation, in contrast to the lasting effect of MSCs, severe withdrawal syndrome appeared when DEX was terminated completely, in the short-term therapy. On day 12, inflammation of DEX-treated rats significantly increased, which led to a new maximum peak of disease (2.2 ± 0.32 vs. 2.9 ± 0.36 [the peak score of the first attack], $t = 3.13$, $P = 0.006$; Figs. 3A, 3B, 3E). In the tapering DEX therapy group, although the dosage was decreased gradually every 10 days, withdrawal symptoms, such as recurrent intraocular inflammation, still occurred (Fig. 3F).

FIGURE 5. The MSC treatment inhibited T cells and APCs in the spleen. T cells from spleens were mixed with APCs, and stimulated with R16 at the indicated concentrations. T cell proliferation was measured at 64 hours by [3 H]-thymidine uptake, and expressed as cpm $\pm$ SD of triplicate wells for each condition. T cell response to R16 was significantly inhibited when T cells and APCs were from MSC-treated rats (A), and also significantly reduced when T cells were from untreated rats and APCs were from MSC-treated rats (B), or vice versa (C). $n = 6$, $*P < 0.05$.

Compared to the control group, the DEX treatment groups did not show any significant long-term amelioration, based upon the clinical scores and incidences within 50 days, while the incidences of the short course DEX group increased. Thickness of the retina and ONL also showed no differences in the tapering DEX treatment group, and were reduced correspondingly in the short-term DEX treatment group compared to those in the control group (Fig. 4). Regarding histological evaluations, all of the three MSC regimens showed better results compared to the DEX treatment results.

MSCs Simultaneously Inhibited the Proliferation Ability of T Cells and the Function of APCs

In vitro, proliferation of splenic T cells from control group rats were correlated positively with concentration of R16. The MSC treatment significantly reduced the responses of splenic T cells to R16 (Fig. 5A). Crossover experiments were designed to analyze further the inhibitory effects of MSCs on T cells and APCs. The APCs from MSC-treated rats led to a less proliferation of T cells from untreated EAU rats (Fig. 5B), and with APCs from the control group rats, the T cells of MSC-treated rats also showed lower proliferative potential (Fig. 5C), which suggested that MSC therapy inhibited the proliferation ability of T cells and the function of APCs.

MSC Treatment Reduced Th1 and Th17 Responses While Upregulating Tregs in rEAU

To determine the effects of MSC treatment on Th1 cells, Th17 cells, and Tregs, we assessed the frequency of CD4⁺IFN- γ ⁺, CD4⁺Foxp3⁺, and CD4⁺IL-17⁺ cells in eyes, lymph nodes, and spleens, on day 10 after transfer, with and without MSC treatment. As shown in Figure 5, except for the frequencies of CD4⁺IFN- γ ⁺ cells, showing a reduction, but without statistical significance in lymph nodes, CD4⁺IFN- γ ⁺ and CD4⁺IL-17⁺ cells were significantly downregulated in eyes, lymph nodes, and spleens, while CD4⁺Foxp3⁺ cells were upregulated after MSC treatment (Fig. 6).

The levels of Th1, Th17, and Tregs cytokines in culture supernatants and AqH paralleled those of T cell subset frequencies. In the conditioned media, production of Th17 and Th1 cytokines, IL-17, and IL-2 significantly decreased in T cells from MSC-treated rats. In contrast, the concentration of the Treg cytokine IL-10 increased compared to cultures from

the control group. In AqH, expression of IL-17, IFN- γ , and TNF- α were reduced significantly, while IL-10 was enhanced in the MSC treatment group (Fig. 7). The levels of expression of IL-2 in AqH and TNF- α in culture supernatants were undetectable.

DISCUSSION

The MSCs are a population of adult stromal progenitors of mesodermal lineage from various sources, which currently are thought to be derived from pericytes, functioning as surveyors of the local environment and responding to injury by sensing and secreting chemokines locally in many body tissues.¹⁸ Given their availability, versatility, immunosuppressive properties, and low immunogenicity, therapies with autologous or allogeneic MSCs have been tested with promising results in a variety of autoimmune diseases, including systemic lupus erythematosus (SLE),⁷ Crohn's disease,⁸ multiple sclerosis (MS),⁹ rheumatoid arthritis,¹⁰ and Sjögren's syndrome.¹¹

In mEAU of rats, we previously demonstrated that MSC therapy remarkably reduced the severity of mEAU when administered before disease stabilization, and nearly completely suppressed the clinical expression of uveitis, and delayed the onset of the disease, when administered preventively.^{14,15} It also has been reported that MSCs can ameliorate mouse EAU by inducing Tregs.¹⁹ Because most types of sight-threatening autoimmune uveitis are chronic and recurrent, in this study we tested the efficacy and identified the proper regimen of MSC therapy using the rEAU model. Lasting clinical efficacy from one course of MSC therapy, administered after the disease was established, was demonstrated by a decreased incidence, reduced mean clinical score, and reduced retinal impairment after 50 days of observation, while two courses of treatment on D4 and D15 offered no additional benefit to the single course of treatment given on D4. However, in contrast to the results of the mEAU experiments, preventive treatment proved to be less efficient than delayed treatment, based upon the clinical scores of the first disease attack and the final histopathologic results, indicating a different mechanism of pathogenesis between two EAU models induced by active and adoptive immunity, respectively.

Long-term effects of MSCs also have been reported in other experimental and clinical studies. A single application of MSCs decreased the severity and number of inflammation relapses in the relapsing-remitting model of experimental autoimmune encephalomyelitis (EAE).²⁰ The lasting therapeutic effect of

FIGURE 6. The FACS analysis of T cell subsets from eye, lymph node, and spleen. The frequency of CD4⁺IFN- γ ⁺, CD4⁺Foxp3⁺, and CD4⁺IL-17⁺ cells in the eye, lymph node, and spleen were assessed on day 10 after transfer. Representative FACS results are shown in (A). In eyes (B) and spleens (D), after MSC treatment, CD4⁺IFN- γ ⁺ and CD4⁺IL-17⁺ cells were significantly downregulated, while CD4⁺Foxp3⁺ cells were markedly upregulated. In lymph nodes (C), the frequencies of CD4⁺IFN- γ ⁺ and CD4⁺Foxp3⁺ cells also were significantly regulated by MSC treatment in the same manner, while CD4⁺IFN- γ ⁺ cells also showed a trend of reduction, but without statistical significance. $n = 6$, * $P < 0.05$.

MSCs also was confirmed in clinical trials of MSC therapy in MS,²¹ SLE,^{21–24} and GVHD.²⁵ In a recent report of SLE, a single course of MSC administration led to long-term relief of the disease, with reduced relapses, while double transplantations failed to enhance therapeutic effects.²⁵ The potential for MSCs to induce long-lasting suppression had been investigated earlier in mixed lymphocyte reaction systems.²⁶ Analysis of the cell cycle showed that T cells, stimulated in the presence of MSCs, were arrested at the G1 phase, and the proliferation potential could not be restored after MSCs were removed from the cultures. This observation may partially explain the paradoxical longevity of immune modulation, despite the short half-life of infused MSCs, in vivo. Our data suggested a long-term immunosuppressive effect of MSC transplantation in the

early stage of the disease, in a rat REAU model. This could contribute to the development of a clinical MSC therapeutic regimen for chronic and recurrent uveitis.

Corticosteroids have been the first line therapy for ocular immune diseases since their introduction. However, a wide range of systemic and ocular complications associated with chronic use of corticosteroids limit their clinical utility, particularly in children. Consistent with clinical experiences and previous studies using the mEAU model,²⁷ the effects of DEX were characterized as being short lasting in REAU, with the inflammation relapsing shortly after the termination of DEX treatment. In tapering DEX therapy, with the dosage decreased gradually to avoid the death of the rats and to mimic the clinical use of corticosteroids, relapses associated with DEX

FIGURE 7. Effects of MSCs on cytokine production in conditioned media and aqueous humor. In the conditioned media (A), the productions of IL-17 and IL-2 by T cells from MSC-treated rats significantly decreased, and the concentration of IL-10 markedly increased. In aqueous humor (B), the expression of IL-17, IFN- γ , and TNF- α significantly decreased, while IL-10 markedly increased in the MSC treatment group.

reduction also were observed and were accompanied by a lasting mild-to-moderate degree of inflammation between each recurrence. Regarding clinical and final histopathologic results, MSCs showed equivalent therapeutic efficacy as DEX in the first acute attack of the disease, and showed significant anti-inflammatory activity superior to DEX in tapering follow-ups. These results may be attributed to the inappropriate DEX tapering method for this rEAU model, which also is one of the dilemmas encountered clinically. The rats treated with the tapering DEX protocol severely lost weight at the end of the experiment (data not shown), and dosage of DEX was difficult to maintain at a high level. Further studies are needed to establish an optimum tapering protocol for DEX, to compare the efficacy of single and combined therapy with MSCs and DEX.

Although the mechanism underlying the immunosuppressive effects of MSCs has not been fully determined, it has been established that via paracrine and cell-cell contact, MSCs can create an immunomodulatory microenvironment, consisting of cytokines,²⁸ cell surface proteins,^{29–31} and exosomes,^{32–34} to regulate the balance of different subsets of T cells, including Th1, Th2, Th17, and Tregs, and to regulate the functions of other immune cell types, including natural killer cells, DCs, APCs, and B cells.^{3,28} It currently is accepted that Th1 cells and Th17 cells are two major pathogenic T cell subsets in EAU, while Treg cells contribute to the control of the disease.^{35–41} In agreement with our previous results with mEAU, we found that in rEAU, systemic MSC transfusion could simultaneously downregulate Th1 and Th17 responses, while upregulating Tregs, locally and systemically. It recently was reported that IL-22, secreted by Th17 and Th22 cells, may be involved in pathogenesis of inflammatory and autoimmune diseases, including Behcet's disease.^{42–45} Th22 cells characterized by producing IL-22 and TNF- α , but not IL-17, were identified as a new Th cell population with diverse functions in different diseases.⁴⁵ Interleukin-22 itself is pro- or anti-inflammatory depending on different diseases and tissue cells stimulated.⁴⁶ Therefore, the role of IL-22 versus IL-17 in recurrent uveitis should be compared in future studies.

Besides studying the effects on T cell subsets, we also designed a crossover experiment in this study, to investigate the role of MSCs on APCs, and found that, besides the effects on T cells, MSC therapy also could inhibit the function of APCs. It was reported that MSCs could promote DCs, which act as main APCs, to differentiate into a distinct regulatory DC population. Compared to the mature DCs, the regulatory DCs express low levels of maturation markers including CD80,

CD86, and CD40 with low ability to stimulate T cell proliferation.⁴⁷ Our results confirmed that MSCs can exert immunosuppressive effects on different cells of the immune system to provide a more favorable balance, and thereby avoid potential exacerbation of one response caused by specific targeting of the other.

The MSCs-treated rats exhibited much less retina damage and photoreceptor loss compared to the DEX-treated and control groups. We hypothesize that this protective effect could be attributed largely to the immunomodulatory function of MSCs, and also to their neurotrophic and antiapoptotic effects, which have been well defined in EAE,⁴⁸ an animal model of MS similar to uveitis with respect to its autoimmune and final neurodegeneration characteristics.

Taken together, our results have demonstrated that, compared to long-term treatment with DEX, a single course of MSCs therapy was more effective in controlling inflammation, reducing relapses, and protecting the retina in rEAU. The MSC therapy inhibited Th1/Th17-mediated inflammation, regulated the balance between Th17 and Tregs, and suppressed the function of APCs. These results suggested that MSCs could be corticosteroid-sparing agents, and may provide a better alternative with fewer side effects and longer lasting suppressive effects for chronic and recurrent uveitis. Further preclinical investigations should clarify the mechanism of the longevity of immune suppression, and the interaction of MSCs and traditional immunosuppressive agents. Because no significant adverse effects of MSC treatment in other autoimmune diseases have been reported in clinical studies to our knowledge, translating MSC therapy into a clinical setting of uveitis is feasible and should help to better understand MSC-based therapies and expand their potential utility.

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