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Original Research

Mesenchymal stem cell derived exosomes-based immunological signature in a rat model of corneal allograft rejection therapy

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Abstract

Background: Mesenchymal stem cells (MSCs) are promising candidates for immunomodulatory therapy that are currently being tested in corneal allograft rejection. In this study, we tested the effects of Mesenchymal stem cells derived exosomes in the corneal allograft rejection model. **Methods**: Mesenchymal stem cells derived exosomes (MSC-exo) were collected and characterized. Wistar-Lewis rat corneal allograft rejection models were established. PKH26 labeled exosomes were used for track experiment. Models were randomly separated into four groups and treated with graded doses of exosomes or same volumn of PBS. Corneal grafts were assessed for rejection degree using slit-lamp biomicroscopy. Grafts were examined histologically using hematoxylin-eosin (H-E) staining and immunohistochemically using antibodies against CD4, CD8 and CD25. A comprehensive graft mRNA gene expression array analysis was conducted and checked by real-time polymerase chain reaction (PCR). **Results**: The nanovesicles obtained were expressing exosome specific protein markers CD9, CD63, CD81. The labeled exosomes could be detected in both cornea and anterior chamber two hours after injection.The 10 μg exosomes subconjunctival injection can effectively prolong graft survival time (MST 16.3 $\pm$ 2.5 days). 10 μg exosomes-treated group can inhibit the infiltration of CD4+ and CD25+ T cells. IFN-γ and CXCL11 levels were significantly decreased in grafts obtained from postoperative exosomes-treated rats when compared with controls. **Conclusions**: MSC-exo can cross biological barrier and play better role directly towards target tissue. MSC-exo can effectively prolong grafts survival time. Th1 signaling pathway was significantly inhibited in the exosomes treated group.

Keywords: mesenchymal stem cell; exosomes; cell-free immunomodulatory therapy; corneal allograft rejection

1. Introduction

Corneal transplantation is widely accepted in corneal blindness for visual rehabilitation. However, it is reported that graft rejection occurred in almost 50% of the cases, and the rejection rate of high-risk cornea could reach to 70% to 90% [1]. The mechanism of graft rejection is complicated and multifactor involved, among which immune responses is the predominant reason for graft failure. Current gold treatments for anti-rejection are systemic corticosteroids and immunosuppressants [2]. Yet its effect in high-risk corneal allograft rejection is limited due to its complications such as drug toxicity and life-threatening potential. New therapies to ensure the viability of corneal transplantation are in need.

Mesenchymal stem cells (MSCs) offer great hope and promise in a series of allograft rejection models due to its immunomodulatory effect [3]. Our and other studies have demonstrated that MSCs possess strong immunosuppressive ability, which can inhibit corneal allograft rejection in animal model *in vivo* [4–6]. However, stem cell based therapy is associated with certain disadvantages like poor targeted migration, engraftment and the survival of the transplanted cells. MSCs may play their role via a paracrine action [7]. Recent studies identifying exosomes as the secreted agents, mediating MSCs therapeutic efficacy, which could potentially replace a cell-based drug by a safer biologic based alternative [8]. Several studies have compared the therapeutic effects of MSCs and their extracellular vesicles (EVs) and did not discover significant differences [9,10].

Based on MSCs' effects on immunity on immunity and our previous work, the current effort presented here sought to improve upon these therapeutic results by extending MSCs efficacy and reducing cell based therapeutic risk. In this study, we investigated the immunosuppressive capacity of MSCs-derived exosomes in corneal allograft rejection model and tried to clarify its immunomodulatory mechanism and functional way.

2. Materials and methods

2.1 Animals

Male Lewis rats (6–8 weeks old) and male Wistar rats (4 weeks old and 6–8 weeks old) purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Bei-

jing, China) were housed under pathogen-free conditions. All procedures of experiments 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.

2.2 Culture and identification of MSCs

Approximately 90–110 g weighted male Wistar Rats at 4 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). After being sacrificed and instantly soaked in 75% ethanol for 10 minutes, the femurs and tibias of the rats were collected under sterile conditions and placed in phosphate buffered saline (PBS, Gibco). Needles were used to drill numerous holes on both ends of the bone and the bone marrow was washed out into the PBS by another needle. Samples were centrifuged and suspended in complete culture medium containing Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DF-12, Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen) and 29.2 mg/mL L-Glutamine (Invitrogen). After being centrifuged at 1000 $\times$ g for 5 minutes, the cells were suspended and incubated at 37 °C in humidified air with 5% CO2 and the culture medium were changed every 3 days. Subconfluent (70~80%) MSCs between passages 3 and 5 were switched to medium supplemented with 10% exosome-depleted fetal bovine serum (FBS, Gibco) for 48 h before collecting the culture. FBS was depleted of bovine exosomes by ultracentrifugation at 100,000 $\times$ g for 70 min. In addition, MSCs were identified by their differentiation capacity into adipocytes and osteocytes when cultured *in vitro*, as previously described [11].

2.3 Isolation and identification of exosomes

The supernatant of MSCs from passages 3 to 5 were collected for exosomes isolation. Supernatant fractions collected from 48 h cell cultures were centrifuged at 300 $\times$ g for 10 min. Then, the supernatant was centrifuged at 20,000 $\times$ g for 20 min. Exosomes were then harvested by centrifugation at 100,000 $\times$ g for 70 min. The exosomes pellets were resuspended and collected in 1 mL of sterile PBS. Exosomes were prepared to pass through a 0.22 μm filter and stored at –80 °C until use. Before injection, the protein concentration of exosomes were determined by Protein BCA Assay Kit (Solarbio, China). Exosomes were diluted into different concentrations. 10 μg/100 μL exosomes were prepared for tracking experiments.

For identification of isolated exosomes, the pellets were fixed with 200 μL 2% paraformaldehyde and were sent to Peking University People's Hospital for electronic microscopy. Besides, markers of exosomes: CD63, CD9 and CD81 (Abcam) were analyzed by western blot. Total proteins from the exosomes pellets were extracted in lysis buffer and protein concentration were measured with the Protein BCA Assay Kit (Solarbio, China). Samples were boiled at 99 °C for 10 minutes and loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (4% stacking gel and 12% separating gel), and then transferred to PVDF membrane. Membranes were blocked with 5% non-fat dried milk and incubated in the primary antibodies overnight at 4 °C, the primary antibodies included antibodies to CD63, CD9, CD81. Membranes were incubated with secondary antibodies for 2 h. Immuno-reactive protein bands were visualized by Multispectral Imaging System (Biospectrum AC Chemi HR 410, UVP, LLC, Upland, CA, USA).

2.4 Corneal allograft rejection model (CARM) and exosomes treatment protocols

Corneal allograft rejection model was induced in Lewis recipient rats by transplanting the cornea grafts from Wistar donor rats. Lewis rats were anesthetized sequentially by intraperitoneal injection of chloral hydrate (10%), 0.3 mL/100 g bodyweight. The pupil of the recipient eye was fully dilated by 0.5% tropicamide. The corneal grafts were 3.5 mm in diameter excised of sacrificed Wistar and instantly kept in wet chamber before transplantation. A 3.0 mm-diameter corneal bed was prepared in the right eye of Lewis. The graft was then placed onto the bed and secured with 8 interrupted 10-0 nylon sutures. The anterior chamber was reformed by injection of air bubble.

The models were randomly divided into 4 groups with 6 rats in each group. For treatment, operated eyes of the Lewis rats were administrated with different doses of MSCs derived exosomes (1 μg, 10 μg, 100 μg) in 100 μL PBS, or an equal volume of PBS in control group. After injection, Tobramycin Eye Ointment was placed on the right eye of Lewis rat. Each group received two injections (a) immediately after transplantation and (b) two days post-op (days 0 and 2) (Fig. 1).

2.5 Clinical assessment

The rats were observed post operation by slit-lamp bio-microscope from day 3. The Larkin method [12] based on opacity, edema, and vascularization scoring is used in graft rejection degree assessment. The graft reached rejection level as indicated by an opacity score $>$3 and a total rejection score $>$5. In addition, recipients with anterior chamber bleeding in the surgery, infection and secondary cataract during the observation period were excluded.

2.6 Gene expression array

On day 10, four corneas of each group (PBS-treated and exosome-treated) were collected for gene expression array, which was performed by Genechem Co., Ltd., Shanghai, China. Total RNA was extracted and the quality was monitored using the Agilent 2100 Bioanalyzer. GeneChip 3' IVT Express Kit was used to prepare the am-

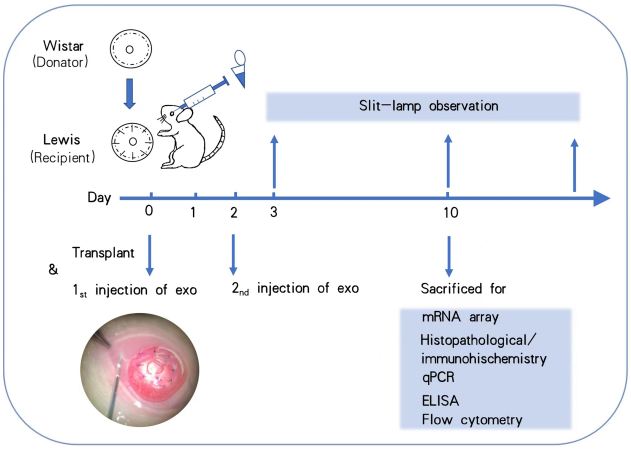

Fig. 1. Rat corneal allograft rejection model. Rejection model was made in a Lewis recipient rat by transplanting the cornea graft from a Wistar donor rat. Injections of exosomes were performed immediately after transplantation and two days post-operation. The slit-lamp observation was performed from day 3 post-operation.

plified RNA (aRNA). After the aRNA was purified and fragmented, it was then hybridized using Genechip Hybridization Oven 645 (Affymetrix, Inc., CA, USA), washed by GeneChip Fluidics Station450 and scanned by GeneChip Scanner 3000. Raw data were analyzed by GeneSpring GX software version 11.5 (Agilent Technologies, CA, USA).

2.7 Histopathological examinations

On day 10, both the PBS-treated group and the exosome-treated group were sacrificed with double-dose of chloral hydrate. The eyeballs (3 eyes per group) were collected and quickly fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA), dehydrated in gradient ethanol (Sigma-Aldrich, St. Louis, MO, USA), embedded in paraffin block, and sagittally sectioned. Continuous sections (4 μm per section) of anterior segment were collected. 5 paraffin sections of the anterior segment containing the thread ends were selected from each eye ball and stained with hematoxylin and eosin (H-E). The stained sections were pictured by BX51 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) with the fixed optical parameters.

2.8 Immunohistochemistry staining

The animals were killed on day 10, eyes were enucleated. Fixed and dehydrated tissues were embedded in paraffin wax and cut into 4 μm sections. After deparaffinization and dehydration, microwave antigen retrieval was performed for 15 min prior to peroxidase quenching with 3% H2O2 in PBS for 15 min. Subsequently, sections were pre-blocked with 5% goat serum albumin for 30 min and incubated with mouse anti-CD4, anti-CD8, anti-CD25 overnight at 4 °C (Mouse anti Rat CD4 (MCA55GA, 1:50, AbD Serotec), Mouse anti Rat CD8 (MCA48GA, 1:500, AbD Serotec), Mouse anti Rat CD25 (MCA273GA, 1:100, AbD Serotec). All of these antibodies and the secondary biotinylated polyclonal goat anti-mouse immunoglobulin were purchased from AbD Serotec. After being washed in PBS, sections were incubated with biotinylated secondary antibody for 30 min, and then stained with 3, 3′ -diaminobenzidine for 3 min. Sections were observed by BX51 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) with the fixed optical parameters. Image J software was used (available on http://rsb.info.nih.gov) semi-automatically for quantification of positive cells from immunohistochemistry section.

2.9 RNA extraction, reverse transcription and quantitative real-time PCR (qPCR)

Grafts of PBS-treated Lewis rats, and exosome-treated Lewis rats (3 rats per group) were collected and frozen in liquid nitrogen on day 10. Total RNA was extracted using Trizol (Thermo Fisher Scientific, CA, USA) under the instruction of the manual. The concentration and purity of total RNA were examined by a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). 1 μg of the total RNA was reverse transcribed using a RevertAid cDNA synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The expression levels of C-X-C motif chemokine ligand 11 (CXCL11), interleukin 2 receptor subunit beta (IL2rb), interferon gamma induced GTPase (IGTP), interferon gamma (INF-γ), T-cell receptor beta chain, and GAPDH genes were detected by quantitative real-time PCR (qPCR) in a HT7900 Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). The reaction mixture contains FastStart Universal SYBR Green Master (Applied Biosystems, Foster City, CA, USA), cDNA template, and gene-specific primers (**Supplementary Table 1**). The program started with 2 min at 50 °C, 10 min at 95 °C, and then followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. To check the amplicon specificity, a dissociation stage was added at last. The relative expression levels of the target genes were analyzed using a comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method. The results were normalized to GAPDH and were carried out in triplicate for each sample.

2.10 Flow cytometry

On day 10, the spleens and lymph nodes were collected from PBS-treated and exo-treated rats. The monocytes were isolated by Ficoll (GE Healthcare Life Sciences, Beijing, China), 1×10^7 was stained with APC-CD4, FITC-CD25 respectively and collaboratively (Biolegend, San Diego, CA); After 20 min incubation on the ice in darkness, the cells were washed 3 times in cell staining buffer (Biolegend, San Diego, CA) and were then incubated with transcription factor fix solution (Biolegend, San Diego, CA) for 30 min in darkness. Then, washed for 3 times by the transcription factor perm buffer followed by staining with PE-FOXP3, and PE Mouse IgG1 antibody for

30 min in the darkness. Following final washes, the cells were fixed with 300 μL staining buffer, and analyzed on the ice for a flow cytometer immediately (BD, NJ, USA).

2.11 Exosomes labeling and tracking

For exosomes' tracking, purified exosomes and PBS were both labeled using PKH26 (red) membrane dye (Sigma-Aldrich) according to the manufacture's protocol. Labeled exosomes and PBS with dye were washed in 40 mL of PBS, collected by ultracentrifugation and re-suspended in 200 μL PBS. Labeled exosomes and PBS with dye were filtered by 0.45 μm filter before exosome injection. Rats after transplantation received the labeled-exosome subconjunctival injection and PBS with dye injection subconjunctival injection as controlled. The eyes were collected after injection for 2 hours and 24 hours. These tissues were frozen-sliced in 6 μm and frozen in –20 ℃. They were taken out and fixed in 4% PFA for 20 min and washed in PBS for three times and stained with 4',6-diamidino-2-phenylindole (Life Technologies, Grand Island, NY, USA) for 20 min before treating. Six random fields were capture per sample at 40 $\times$ magnification, and representative pictures were taken at 200 $\times$ magnification.

2.12 Statistical analysis

All statistics were processed by GraphPad Prism version 6.00 for Mac (GraphPad Software, La Jolla, CA, USA). Results of survival time were assessed by Kaplan-Meier analysis. The relative mRNA level was analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test. Data are expressed as mean $\pm$ SD. $p \leq 0.05$ was considered statistically significant. Results are presented as the mean $\pm$ SEM.

3. Results

3.1 MSC-exo characterization and identification

MSCs were characterized by their multilineage differentiation potentials, including chondrocytes, osteoblasts, and adipocytes. The vesicles of MSCs in our experiments were identified by electron microscopy and the vesicles were cup-shaped and measured 30–150 nm in diameter (Fig. 2A). Western blot confirmed that the vesicles from MSCs expressed markers of exosomes, including CD63, CD9 and CD81 (Fig. 2B).

3.2 Clinical assessment

We have previously reported that conjunctiva injection of MSCs could prevent the rejection of allograft in rats. To explore whether exosomes released from MSCs could inhibit the allograft-rejection, we applied three different doses of MSC-exo (1 μg, 10 μg, 100 μg) in subconjuctival injection route at first. In the PBS group, mean graft survival time (MST) was 9.67 $\pm$ 1.37 days. In the PBS treated group, mean graft survival time (MST) was 9.5 $\pm$ 0.84 days. As seen in Fig. 3A, when the allografts of control rats were

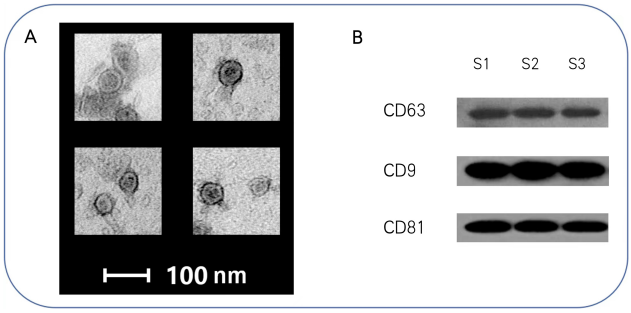

Fig. 2. Identification and characteristics of exosomes. (A) Electron microscopy image of exosome isolated from BMSCs-medium. (B) Western-blot results. CD63, CD9, CD68 were as the markers of exosomes. Three independent samples from vesicles of MSCs (marked as S1, S2 and S3) were performed here.

prone to be rejection around day 10, the survival rate of allografts in 10 μg MSC-exo subconjunctival treatment group (MST was 16.3 $\pm$ 2.5 days) was 100%. However, 1 μg (MST was 9.3 $\pm$ 1.0 days) and 100 μg (MST was 8.7 $\pm$ 1.4 days) exosomes groups did not show any difference in attenuation of rejection (Fig. 3A). Then, we further examined different delivery routines of MSC-exo. Compared to the PBS treatment group, the eye drops group (MST was 10.5 $\pm$ 1.0 days) makes no significant difference in corneal allograft survival ($p > 0.05$). As showed in the Fig. 3B, on day 10, the graft of exo-treated group was still transparent with a visible pupil but the graft of PBS-treated group was opaque with severe edema and new vessels grow into the center of the cornea. 10 μg exosomes subconjunctival-injected was the optimal way for treatment. Exosomes based subconjunctival treatment decreased the tempo of rejection by alleviating the graft edema and neovascularization.

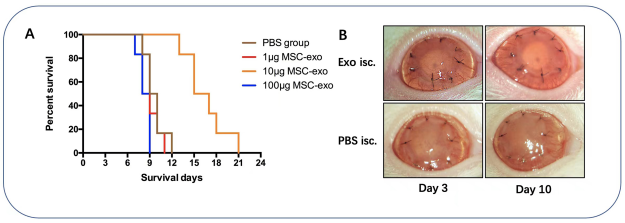

Fig. 3. The role of MSC-exo in corneal allograft rejection. (A) 10 μg MSC-exo treatment prolonged the allograft survival while 1, 100 μg MSC-exo had no effect on survival. (B) slit-lamp images showed that 10 μg BMCs-exo subconjunctival injection after operation alleviated the graft edema and neovascularization at day 3 compared with PBS group and at day 10, the graft of exo-treated group was still transparent with a visible pupil but the graft of PBS-treated group was opaque with severe edema and new vessels growed into center of the cornea (n = 6).

3.3 Tracking of MSC-exo

MSC-exo was labeled by PKH26 to investigate the trace of exosomes after subconjunctival injection. The labeled MSC-exo were detected via confocal laser scanning microscopy (Fig. 4). Two hours after MSC-exo injection, there were numerous exosomes could be detected both in the cornea and anterior chamber. 24 hours after, the fluorescent intensity was weaker and the quantity was markedly decreased. However, there was no positive finding in spleen tissue either 2 hours or 24 hours after exosomes injection.

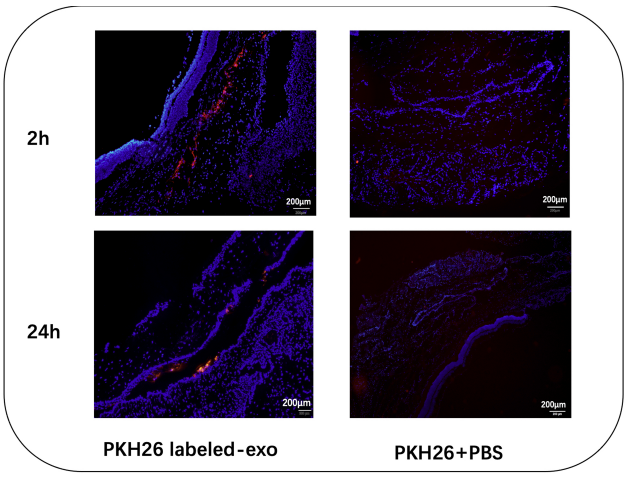

Fig. 4. Tracking of BMSC-exo in sub-conjunctiva. The exosome in the recipient rat's subconjunctiva after 2h/24h administrating PKH26 labeled-exo as the images of left row showed. The controlled group were injected PKH26-incubated PBS as the right column showed. Labeled-exo still can be observed after 24h with a weaker fluorescent intensity (n = 2).

3.4 MSC-exo treatment reduced leukocyte infiltration in the allografts

CD4+, CD8+ and CD25+ cells infiltration were assessed by immunohistochemistry. As shown in Fig. 5, the numbers of CD4+, CD25+ cells in the allografts were decreased in MSC-exo treatment group compared to PBS group (p = 0.007, p = 0.001, respectively). No statistical significance was found in the number of CD8+ cells infiltrating corneal grafts compared to control groups (p = 0.937).

3.5 MSC-exo treatment upregulated Treg cells expression

MSC-exo also up-regulated the proportions of CD4+CD25+Foxp3+cells in the splenocytes and lymph nodes by flow cytometry (Fig. 6). The ratio of Foxp3+expressing Treg cells/CD4+ T cells increased in the MSC-exo group (8.56 $\pm$ 0.72) when compared with PBS-treated group (5.72 $\pm$ 1.14) by flow cytometry.

3.6 Validation of genes and gene enrichment and pathway analysis of mRNA array

To examine the possible mechanisms behind the effectiveness of local MSC-exo therapy, we further performed the mRNA array. Three independent samples were studied for each group. And three duplications were made for an independent sample. Quantitative real-time polymerase chain reaction was performed to confirm the results from mRNA array. Five obviously altered genes, including IFN-γ, IL2R, CXCL11, IGTP, TCR, were examined at day 10 post-op. using qPCR. Enrichment analysis showed that MSC-exo participated in the inhibition of Th1 signaling pathway, Th1 signaling pathway was significantly inhibited (Fig. 7). As demonstrated in Fig. 8, mRNA expression levels of IFN-γ, IL2R, CXCL11, IGTP, TCRß were all significantly decreased, which has the same tendency of the reports of mRNA array, proving the reliability of the mRNA array analyses.

4. Discussion

Penetrating keratoplasty is the last resort for patients who suffer the irreversible blindness caused by corneal diseases. However, the demand of recipients for transplantable corneas outnumbers the supply of donor corneas. Meanwhile, transplanted patients are facing the risk of immune response and graft rejection on a lifelong basis. Despite the advancement of corneal transplantation in recent years, the penetrating keratoplasty has an irreplaceable role in some corneal diseases, especially disorders affecting both the stromal layers and endothelial layers. With regard to the penetrating keratoplasty, the allograft survival is limited though under the administration of immunosuppressants. Although it has been demonstrated that MSCs could promote graft survival by inhibit immune response, cell based treatment has potential uncertainty about safety and efficacy [13]. Exosomes are considered a form of extracellular vesicles with a size ranging from 30 to 150 nm. Exosomes contain cell-derived specific functional components, including proteins, lipids, mRNAs and miRNAs, which enable them to play a role similar as their original cells [14]. The potential advantages of exosomes in cargo delivery including easily sterilized, low to none-immunogenicity for allogenic use, easy manipulation for loading of therapeutic agents, better crossing through the biological barriers, which make them ideal for cell-free therapeutic strategies. In the light of this statement, MSC-exo has been used in a plethora of degeneration and allograft rejection models and has inspiring results [15–17]. Effects of exosomes from different MSCs types are not equivalent, and the quality of exosomes obviously depend on the quality of the secreting stem cells [18,19]. Bone marrow-derived mesenchymal stem cells were used in our research. In this study, the grafts survival time were accessed to be prolonged after MSC-exo treatment. It indicated that the use of exosomes may represent a cell-free alternative approach in corneal allograft

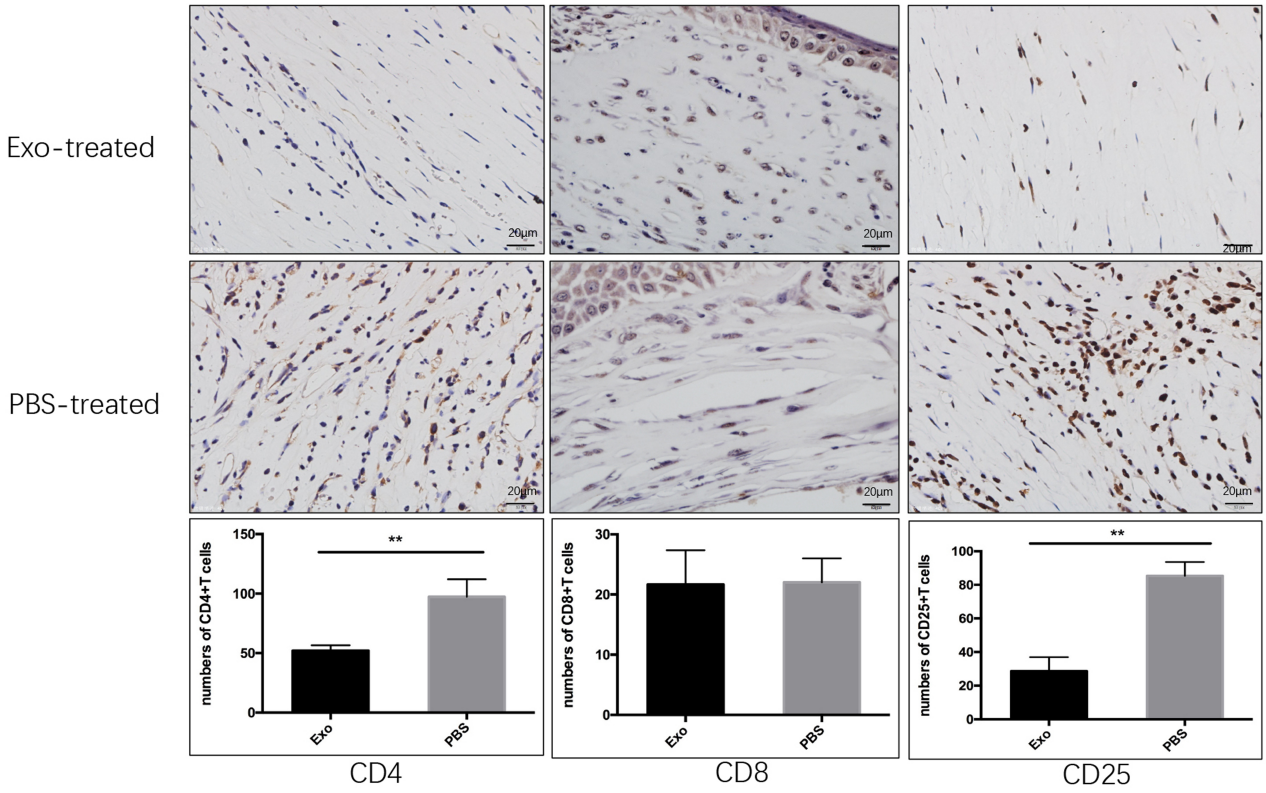

Fig. 5. Representative immunohistochemistry staining of CD4, CD8, and CD25 in corneal allografts. The numbers of the cells positive for CD4, CD8, and CD25 staining were quantified and compared with PBS groups (n = 3). *$p < 0.05$, **$p < 0.01$. Scale bar = 20 μm.

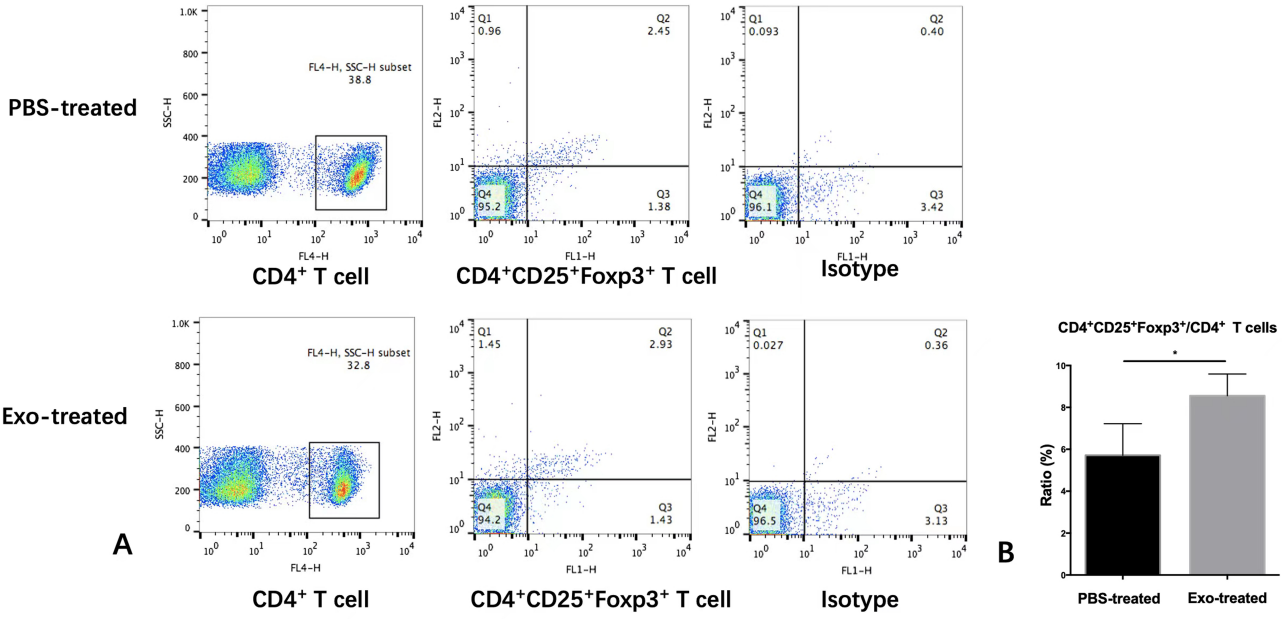

Fig. 6. Flow cytometry was used for analysis of proportion of CD4+CD25+Foxp3+Tregs. Splenocytes from recipient rats were harvested at day 10 after allograft transplantation. Representative splenocytes sample of the percentages of CD4+, CD4+CD25+Foxp3+ T cells were represented. Mean ± SD of the proportions of CD4+CD25+Foxp3+ T cells in CD4+ T cells are shown. Data were collected by three independent experiments. * $p < 0.05$.

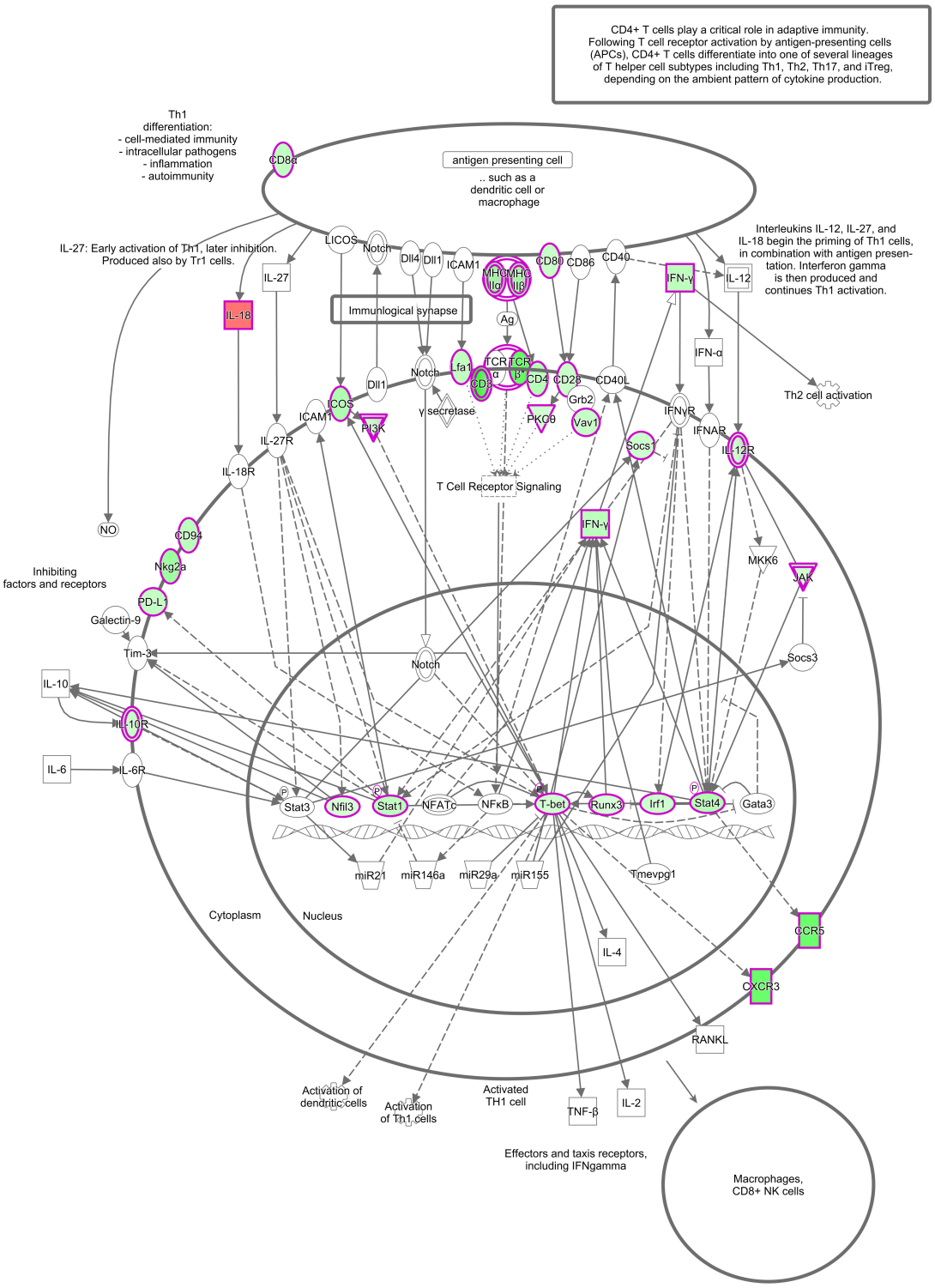

Fig. 7. Th1 pathway map. In this experiment, Th1 pathway was significantly inhibited, compared with **Supplementary Fig. 1**. The green color indicated the gene was significantly down regulated and red color indicated the gene was markedly up regulated.

rejection.

In this study, PKH26-labeled MSC-exo was administrated in the subconjunctiva of corneal allograft rejection model. The results showed that there was a small quantity of MSC-exo which could be observed in subconjunctiva and some of the exosomes could be tracked in allografts as well as anterior chamber. Our previous results showed that MSCs were accumulated in the subconjuctiva after local injection [6], this indicated that MSCs acted via a paracrine way, rather than direct cell contact. This is in accordance with the work of Lin *et al.* [20]. They found that after topical administration of MSCs to the murine corneal epithelial

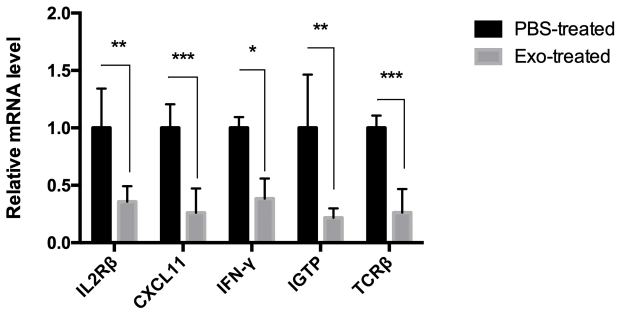

Fig. 8. Verification of relative expression of Th1 cytokine-IFN-γ and other four differentially expressed genes. The results showed that the tendency of these five genes were similar and they were all decreased when comparing the exo-treated group with the PBS-treated group, which was same as the report of mRNA array. (*<0.05, **<0.01, ***<0.001).

wounding model, the corneal stroma rather than epithelium is the retention place of most MSCs. So the attenuating effect to the inflammation and promotion effect to the regeneration of epithelium are explained by soluble factors released from MSCs. Extracellular vesicles, including exosomes can mediate the paracrine effect of MSCs [21–23]. Our results verified that exosomes could cross biological barrier and play better role directly towards target tissue.

Cornea is located at the surface of the eye, which makes it easy to access and manipulate. A few studies have reported the therapeutic effects of MSC-exo on corneal wound models [24,25]. It is reported that human corneal mesenchymal stromal cells derived exosomes promoted murine corneal epithelial wound healing [24]. Topical MSC-exo treatment was also reported could suppress corneal inflammation and corneal scarring [25]. These data showed the potential use of MSC-exo in ocular surface diseases therapy. Local delivery routine is a regular treatment way in ocular disease. Two different delivery routes to treat corneal allograft rejection were compared in this study. The grafts survival observation results showed that subconjunctival injection, other than topical application, was effective. In this study, the failure of eye drops therapy may be due to frequently blink, rapid tear turnover as well as low drug bioavailability [5]. Previous studies showed that exosomes might play their roles in a dose-dependent way. Graded doses of exosomes derived from MSCs were applicated in the study. The results showed 10 μg MSC-exo reached effective dose therapy group can prolong the graft survival time. The highest dosage of MSC-exo treatment group didn't get a better effect as we expected. The grafts in this group were prone to be edema after injection compared to other groups. It is known that the intended biological effects of exosomes can only be uptake by target cells in an endocytosis pattern [26]. We assumed that maybe the dose exceeded the target cells uptake capacity or due to possible contamination with apoptotic bodies [18]. Indeed, much is still unknown with regard to which components of the exosomal cargo are responsible for the various observed effects.

Corneal allograft rejection is a form of delayed-type hypersensitivity (DTH) response, which predominantly mediated by CD4+ T cells. CD4+ T cells have the potential to differentiate into different subgroups. Among the numerous subsets, the helper T-cell (Th) and regulatory T-cell (Treg) subtsets are the best characterized. Treg plays a key role in immune tolerance maintaining in corneal allograft rejection [27]. It's interesting that the proportion of Foxp3 expressing CD4+CD25+ regulatory T cells were found increased in the MSC-exo treated group. This result suggest that MSC-exo therapy can induce immune tolerance by upgrading the expressing of Tregs. The homeostasis of CD4+Th cells subtypes plays key roles in the pathogenesis of allograft rejection. In corneal allograft rejection T-cell differentiation is primarily polarized toward the Th1 response, driven by pro-inflammatory cytokines, particularly IFN-γ. Th1 cells are characterized by secretion of IL-2 and IFN-γ which provide a positive feedback loop stimulating further proliferation of Th1 cells . The CD4+T cells were attracted by the inflammatory site and secreted IFN-γ associated chemokines CXCL9, CXCL10 and CXCL11. Th1/Th2 balance is important in allograft rejection. However, CD4+ Th1 lymphocyte differentiation produced a large quantity of IFN-γ, which shifted the Th1/Th2 balance toward Th1 population. In the present study, the mRNA sequence revealed that Th1 signaling pathway was significantly inhibited in MSC-exo treated cornea, and we further confirmed that the level of the IFN-γ inducible chemokines CXCL9, CXCL10, and CXCL11 was decreased, especially CXCL11. Chemokines play a primary yet vital role in regulating the migration of immune cells. These results suggest that MSC-exo therapy can inhibit Th1 response in the local area, and thereby block the strong recruitment and retention of leukocytes.

5. Conclusions

To sum up, our results indicated that subconjunctival injection of MSC-exo can prolong corneal allograft survival by inhibiting Th1 response. These findings indicate that local MSC-exo therapy is a promising alternative method for the prevention and treatment of immune rejection after corneal transplantation.

Author contributions

ZJ and SZ designed the research study. ZJ, YL and WZ performed the research. SZ performed the transplant operations and revised the manuscript. XZ revised the manuscript. XZ, FL and XL provided help and advice on research performed. ZJ and YL analyzed the data. ZJ and YL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures of experiments involving rats were approved by the Laboratory Animal Care and Use Committee of Tianjin Medical University (permission number: SYXK2009-0001), and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/27/3/10.31083/j.fbl2703086.

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