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Netrin-1 prolongs skin graft survival by inducing the transformation of mesenchymal stem cells from pro-rejection to immune-tolerant phenotype

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Abstract. – OBJECTIVE: Mesenchymal stem cells (MSCs) induce allograft immune tolerance, but low efficacy severely limits their wide application. In this work, Netrin-1 was used to maintain MSC function in an IR environment to study its role in the immune tolerance induction of the allograft.

MATERIALS AND METHODS: The experiments were divided into three groups: the control group, the IR group and the Netrin-1 group (Netrin-1 was added to MSC medium and then cultured for 48 h). After digestion, MSCs were mixed with TLR4 and TLR3 antibodies (BD), incubated for 20 min, and washed with Phosphate-Buffered Saline (PBS) three times. The mean fluorescence intensity (MFI) of TLR4 and TLR3 was detected by flow cytometry. Isolated lymphocytes were divided into four groups: the control group (no treatment), the MSC group (lymphocytes were co-cultured with MSCs in the control group), the rejection group (lymphocytes were co-cultured with MSCs in the IR group), and the Netrin-1 group (MSCs in the IR group) was stimulated by Netrin-1 for 48h.

RESULTS: Our study found that compared with control mice, toll-like receptor (TLR3) expression in bone marrow MSCs decreased as the expression of TLR4 increased, the secretion of transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) was reduced, while the secretion of IL-6 significantly increased in immune rejection (IR) mice. MSCs in IR mice promoted T-cell proliferation and reduced the ratio of Treg cells. Netrin-1 inhibited the pro-rejection effect of these MSCs, further inhibited T-cell proliferation and facilitated an increase in the ratio of Treg cells. The animal experiment results showed that MSC transplantation in the rejection group would shorten the mean survival time of the skin graft and induce the infiltration of lymphocytes. Netrin-1 prolonged the mean survival time of the skin graft by enhancing MSC function. The immunohistochemistry results showed that, compared with the rejection group, the T cell number in the skin graft significantly decreased in the Netrin-1 group.

CONCLUSIONS: MSC can be divided into immune-tolerant and pro-rejection types in organ transplantation and Netrin-1 can induce the transformation of MSC from the pro-rejection to immune-tolerant type and markedly prolong the skin graft survival time.

Key Words:
Mesenchymal stem cell, Transplantation, Netrin-1, Immune tolerance.

Introduction

Organ transplantation is the most effective treatment for end-stage organ diseases, and rejection is the primary independent risk factor that affects the long-term survival of transplanted organs. Immunosuppressive agents significantly reduce the incidence of immune rejection (IR), but it may be accompanied by many complications, such as infection and tumorigenesis[1]. In recent years, scientists have been seeking a new way to induce immune tolerance; regulatory cell transplantation is considered one of the most promising alternatives[2,3].

A mesenchymal stem cell (MSC) is a kind of adult stem cell that can induce allograft immune tolerance by inhibiting T-cell proliferation and optimizing the local immune microenvironment[4]. MSCs increased the 4-year survival rate of a transplanted kidney by secreting immunosuppressive molecules[5]. Peng et al[6] found that do-

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nor-derived MSCs markedly reduced the use of immunosuppressive drugs and effectively maintained the long-term survival of a transplanted kidney. However, the role of MSCs inducing the immune tolerance *in vivo* is not significant, and large amplification *in vitro* is needed before treatment.

Nerve molecules play a crucial role in revascularization, immunoregulation and tissue regeneration. Netrin-1 is a secretory axon guidance molecule that plays an important role in the reconstruction and repairing of neural pathways[7]. It was found that Netrin-1 inhibited the migration and recruitment of inflammatory cells through the mitogen-activated protein kinase (MAPK) and eukaryotic protein kinase (ERK) pathway; it also inhibited excessive local inflammatory response[8,9]. In addition, Netrin-1 maintained MSC function under ischemic conditions and promoted the reconstruction of nerves and blood vessels[10]. In this work, Netrin-1 was used to maintain the MSC function in an IR environment to study its role in the immune tolerance induction of the allograft.

Materials and Methods

Skin Transplantation

All animal experiments were performed in accordance with the regulations on animal experiments of the Eighth Medical Center of the PLA General Hospital (Beijing, China) and were approved by the hospital's Ethics Committee. After anesthesia by pentobarbital sodium, the Balb/c mice back skin was taken and transplanted to the backs of C57BL/6 mice. Interrupted sutures were carried out with the operation line in the connection of the skin, and the mice were reared under suitable conditions. The judgment of skin allograft survival was defined by Pilon et al[11]: Skin grafts were monitored daily by visual and tactile examination. The rejection was defined as greater than 80% graft necrosis.

Isolation and Identification of Bone Marrow MSCs

MSCs were isolated from the femurs of Balb/c mice. After removing the ends of the femurs, the bone marrow residues were rinsed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and then centrifuged at 1500 rpm for 20 min. After washing them three times, BM-MSCs were cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) medium (Gibco, Grand Island, NY, USA) plus 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Forty-eight hours later, the unattached cells were removed and the remaining cells continued to be cultured. Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse stem cell antigen (Sca)-1, CD11b, phycoerythrin (PE)-conjugated rat anti-mouse CD29, CD44, CD34 and CD45 were used for flow cytometry analysis to identify the mouse MSCs.

Detection of MSC Phenotype

The experiments were divided into three groups: the control group, the IR group and the Netrin-1 group (Netrin-1 was added to MSC medium and then cultured for 48 h). After digestion, MSCs were mixed with TLR4 and TLR3 antibodies (BD), incubated for 20 min and then washed with PBS three times. The mean fluorescence intensity (MFI) of TLR4 and TLR3 was detected by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

After culturing for 48 h, the MSC medium in each group was collected and centrifuged at 3000 rpm for 5 min; then, the supernatant was collected. The transforming growth factor-β (TGF-β), interleukin-10 (IL-10) and IL-6 concentrations in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA; Neobioscience, Shenzhen, China).

Cell Co-Culture

At 48 h after skin transplantation, the heart blood of mice was collected and then lymphocytes were isolated. Isolated lymphocytes were divided into four groups: the control group (no treatment), the MSC group (lymphocytes were co-cultured with MSCs in the control group), the rejection group (lymphocytes were co-cultured with MSCs in the IR group), and the Netrin-1 group (MSCs in the IR group was stimulated by Netrin-1 for 48 h. After being washed with PBS three times, MSCs were co-cultured with lymphocytes). After culturing for 48 h, the lymphocytes were collected. We used the mouse regulatory T cell staining kits (eBioscience 88-8111-40, San Diego, CA, USA) for the instructions and flow cytometry to detect the ratio of $CD4^+CD25^+Foxp3^+$Treg cells.

Cell Proliferation Experiment

To study the effect of MSC on T-cell proliferation, an EDU proliferation experiment was carried out. Cell grouping was the same as be-

fore. MSCs were cultured with lymphocytes for 24 h, T cells were sorted by magnetic beads and a 5-Ethynyl-2'- deoxyuridine (EdU) staining reaction solution (R&D Systems, Minneapolis, MN, USA) was added and then cultured for 24 h. The supernatant was discarded and the cells were washed with PBS three times. Then, 0.5% TritonX-100 was added and penetrated for 10 min; then, a staining reaction solution was added and incubated in a dark place for 30 min. After fully washing with 0.5% TritonX-100, it was stained with DAPI for 5 min, washed with PBS three times, section sealed and observed under a fluorescence microscope.

Animal Experiment

C57BL/6 mice were randomly divided into four groups: the control group (prior to skin transplantation, saline was injected), the MSC group (prior to skin transplantation, 2×10^6 MSCs in the control group were injected from the caudal vein), the rejection group (prior to skin transplantation, 2×10^6 MSCs in the IR group were injected from the caudal vein), and the Netrin-1 group (prior to skin transplantation, 2×10^6 MSCs in the Netrin-1 group were injected from the caudal vein). The transplanted skin was removed after 7 days and fixed in 4% paraformaldehyde. Then, a paraffin section was performed for hematoxylin and eosin (HE) staining (Boster, Wuhan, China) and immunohistochemistry.

Statistical Analysis

The *t*-test and variance analysis were performed using Statistical Product and Service Solutions (SPSS) 12.0 software (SPSS Inc., Chicago, IL, USA). All experimental data were expressed as means ± standard deviation. A *p*-value of less than 0.05 was considered statistically significant.

Results

Isolation and Identification of Mouse BM-MSCs

After 48 hours of culturing, non-adherent cells were removed and adherent cells were selected. The MSCs were expanded to six generations and then digested by trypsin. Surface markers were identified by flow cytometry. We found the BM-MSCs were $CD45^-CD11b^-CD34^-Sca\text{-}1^+CD44^+CD29^+$ cells (Figure 1), consistent with the previous report[12].

Impaired Immune Tolerance of MSCs in IR Mice

Waterman et al[13] reported that TLR4-primed MSCs secreted more proinflammatory molecules and TLR3-primed MSCs secreted more immunosuppressive molecules. Svobodova et al[14] also found that the levels of MSC-secreted TGF-β and IL-6 would affect naive T cell differentiation into Treg cells or proinflammatory Th17 cells.

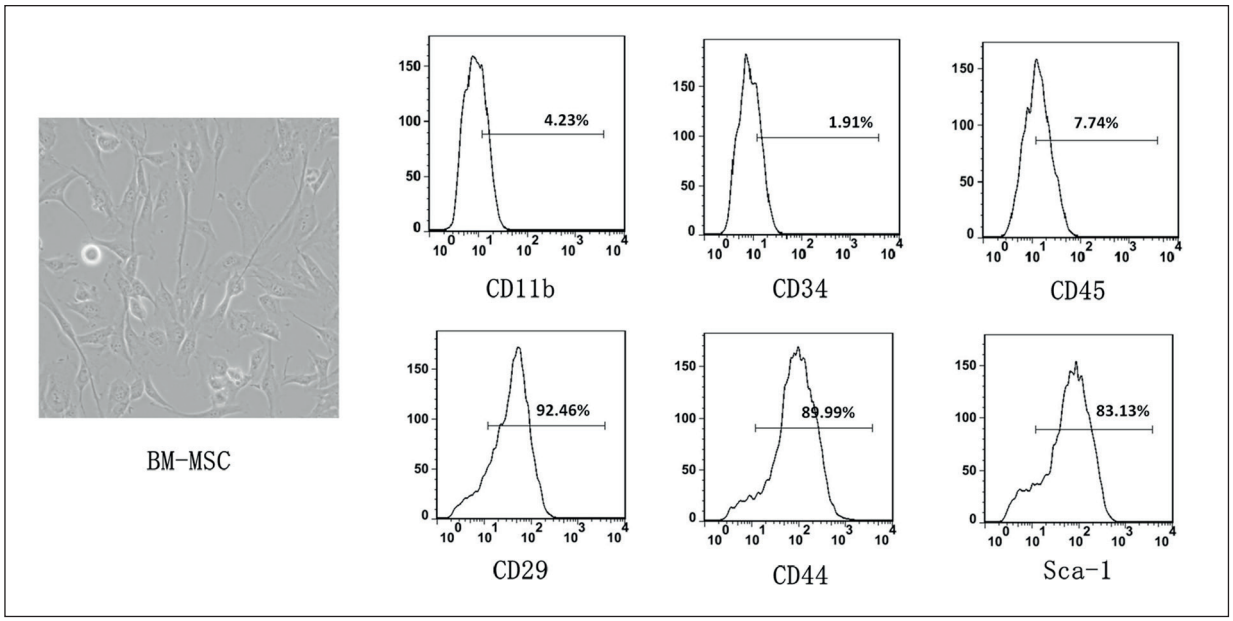

Figure 1. The mesenchymal stem cells extracted from mouse bone marrow are typically spindle-shaped (magnification: 40×). The surface molecular expression is $CD29^+CD44^+Sca\text{-}1^+CD34^-CD45^-CD11\text{-}b^-$.

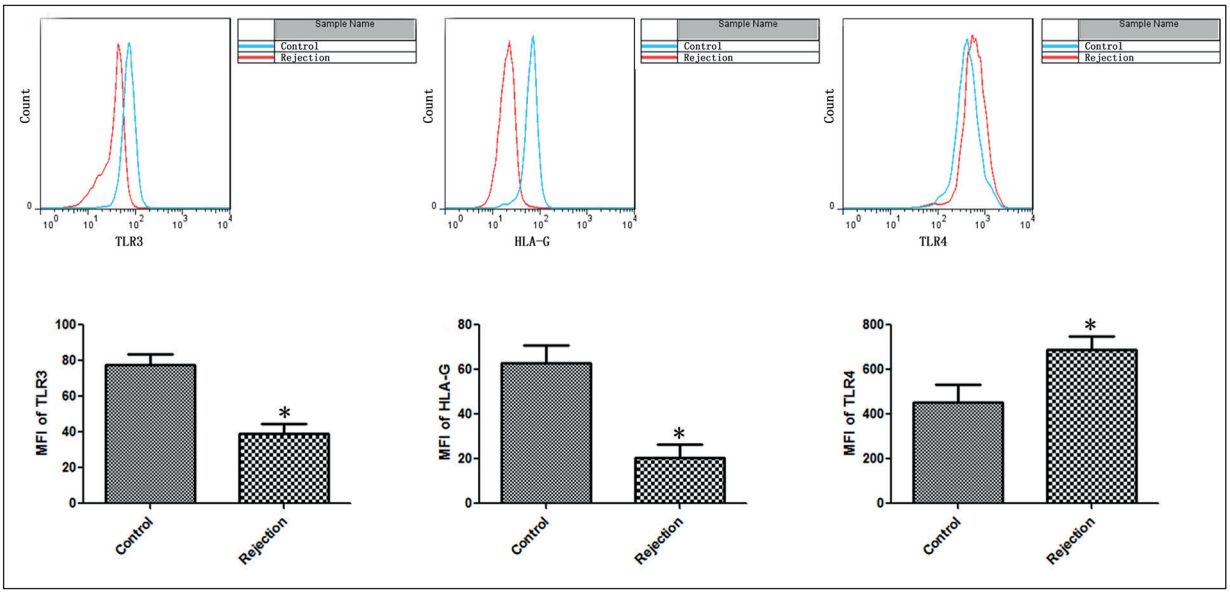

Figure 2. The expression of immunosuppressive protein on MSC in the rejection group significantly decreased. Compared with the control group, mean fluorescence intensity (MFI) of TLR3 decreased by 49.7%. Compared with the control group, MFI of TLR4 increased by 35.1%. *p<0.05 (n=10) *vs.* Control. Values are mean ± SD.

Therefore, pro-rejection MSCs and immune-tolerant MSCs can be identified by detecting the expression of TLR4 and TLR3 on the surface of MSC and the levels of MSC-secreted TGF-β, IL-10, and IL-6.

Flow cytometry showed that immunosuppressive protein expression in MSCs in the rejection group significantly decreased. Compared with the control group, the MFI of TLR3 decreased by 49.7%, while that of TLR4 increased by 35.1% (Figure 2); the ELISA results showed that the secretion of immunosuppressive molecules from MSCs in the rejection group markedly decreased. Compared with the control group, the concentration of TGF-β and IL-10 in the supernatant decreased by 45.1% and 39.6%, respectively, while that of IL-6 increased by 1.7 times (Figure 3).

Netrin-1 Facilitated Immunosuppressive Protein Expression in MSCs in the Rejection Group

Netrin-1 significantly facilitated the expression of the immunosuppressive protein in MSCs in the rejection group. Compared with the rejection group, the MFI of TLR3 increased by 2.58 times, while that of TLR4 decreased by 21.4% (Figure 4).

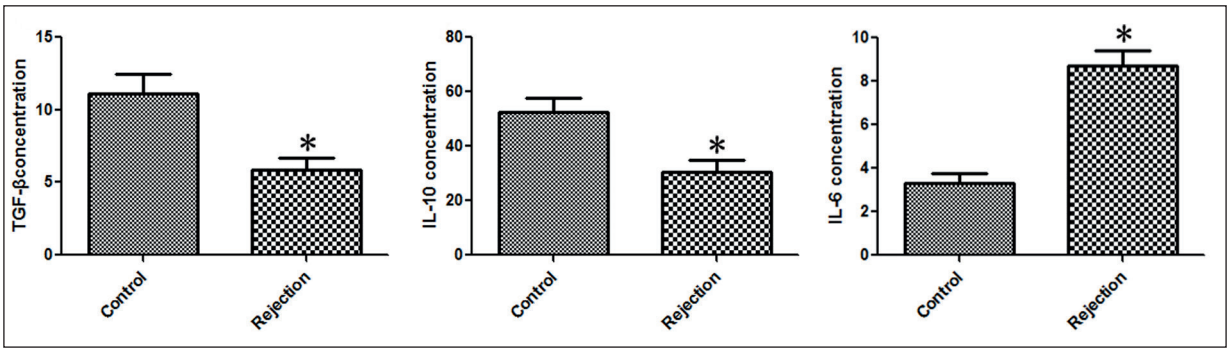

Figure 3. The secretion of immunosuppressive molecules from MSC in the rejection group markedly decreased. Compared with the control group, the concentration of TGF-β and IL-10 in the supernatant decreased by 45.1% and 39.6% respectively, while that of IL-6 increased by 1.7 times. *p<0.05 (n=10) *vs.* Control. Values are mean ± SD.

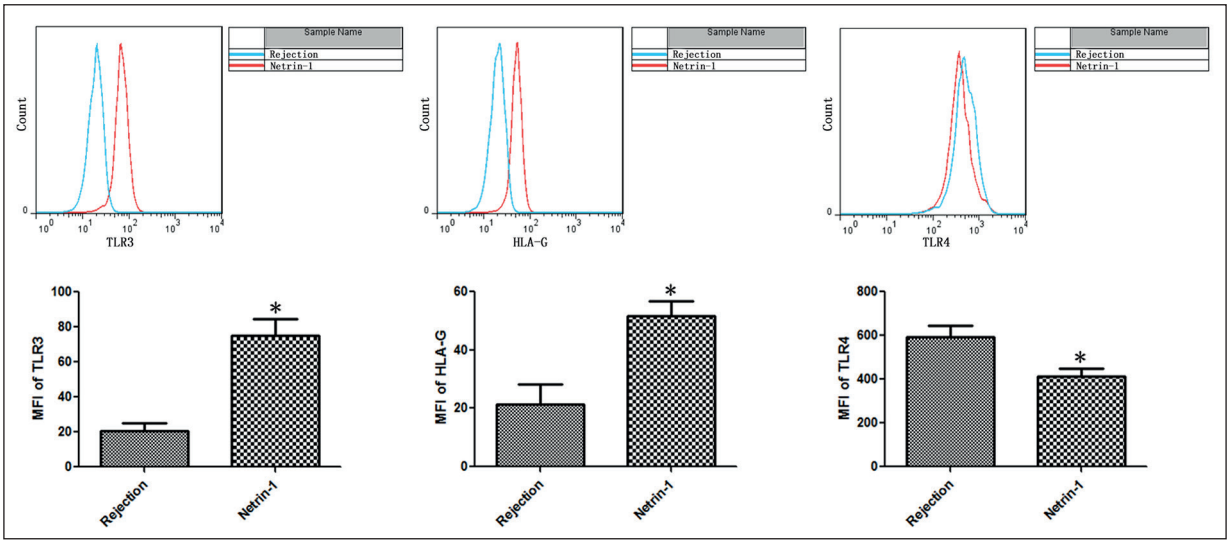

Figure 4. Netrin-1 facilitated the expression of immunosuppressive protein on MSC in the rejection group. Compared with the rejection group, MFI of TLR3 increased by 2.58 times. Compared with the rejection group, MFI of TLR4 decreased by 21.4%. *p<0.05 (n=10) *vs.* rejection. Values are mean ± SD.

Netrin-1 Promoted the Secretion of Immunosuppressive Molecules from MSCs in the Rejection Group

Compared with the rejection group, the concentration of TGF-β and IL-10 in the Netrin-1 group increased by 84.6% and 122.7%, respectively, while that of IL-6 decreased by 49.6% (Figure 5).

Netrin-1-Stimulated MSCs Inhibited T-Cell Proliferation

The EDU results showed that MSC markedly inhibited T-cell proliferation. Compared with the MSC group, the proliferation rate of T cells in the rejection group increased by 4.27 times; compared with the rejection group, the proliferation rate of T cells in the Netrin-1 group decreased by 56% (Figure 6).

Netrin-1-Stimulated MSCs Promoted the Increase of Treg Cells

$CD4^+CD25^+Foxp3^+$Treg cells play an important role in MSC-induced T-cell immune tolerance[15]. Our results showed that, compared with the control group, the Treg cell ratio in the MSC group increased by 3.21 times; the Treg cell ratio in the rejection group significantly decreased, and it decreased by 96.5% compared with the MSC group; compared with the rejection group, the Treg cell ratio in the Netrin-1 group increased by 10.3 times (Figure 7).

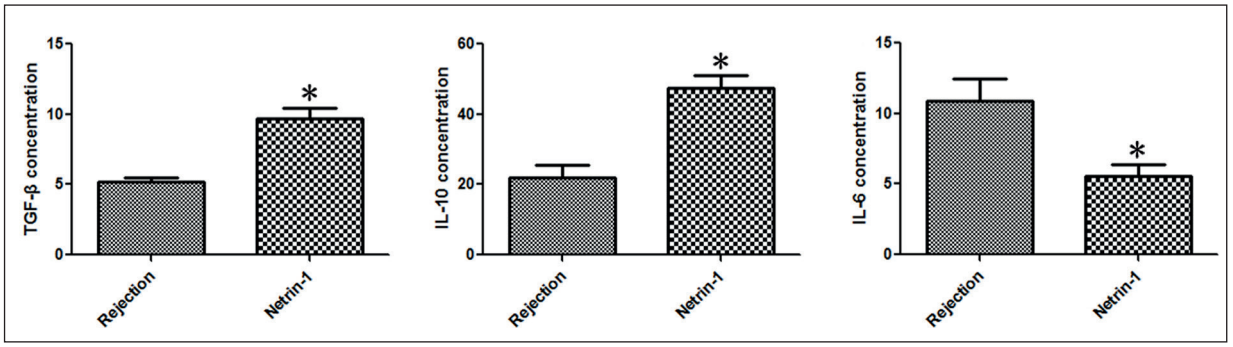

Figure 5. Netrin-1 promoted the secretion of immunosuppressive molecules from MSC in the rejection group. Compared with the rejection group, TGF-β and IL-10 concentration in the supernatant increased by 84.6% and 122.7% respectively, while that of IL-6 decreased by 49.6%.*p<0.05 (n=10) *vs.* rejection. Values are mean ± SD.

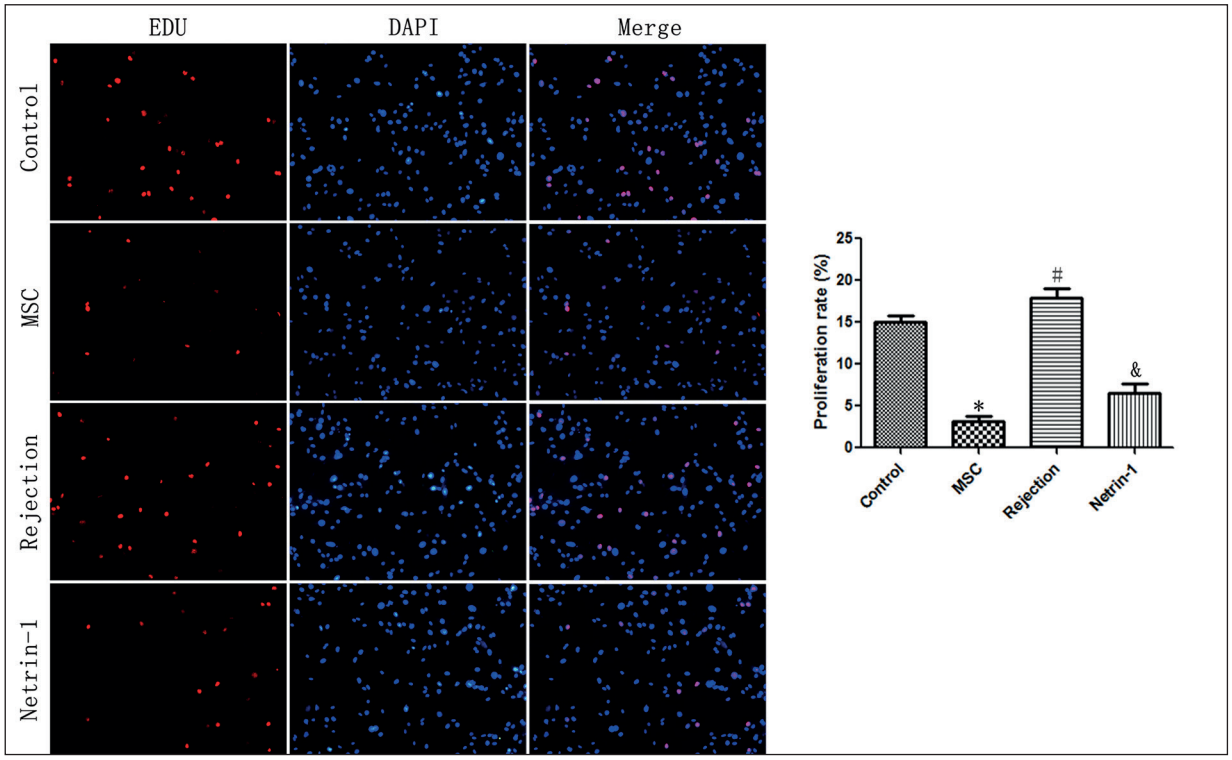

Figure 6. Netrin-1-stimulated MSC inhibited T cell proliferation (magnification: 40×). Compared with the MSC group, proliferation rate of T cells in the rejection group increased by 4.27 times. Compared with the rejection group, proliferation rate of T cells in the Netrin-1 group decreased by 56%.*p<0.05 (n=6) *vs.* Control; $^{\#}p$<0.05 (n=6) *vs.* MSC; $^{\&}p$<0.05 (n=6) *vs.* rejection. Values are mean ± SD.

Netrin-1-Stimulated MSCs Prolonged Survival Time of Skin Graft

The mean survival time of the skin graft in control and MSC groups was 9.6 and 14 days, respectively. The mean survival time in the rejection group was only 7.6 days, which was remarkably shorter than that in the MSC group. The mean survival time of the skin graft in the Netrin-1 group was 14.8 days, which was significantly longer than that in the rejection group. The HE staining results showed that after 7 days of skin transplantation, compared with the MSC group, the lymphocyte number of the transplanted skin in the rejection group markedly increased; Netrin-1-simulated MSCs can significantly inhibit the infiltration of lymphocytes (Figure 8).

Netrin-1-Stimulated MSCs Inhibited the Infiltration of T Cells

The ratio of $CD3^{+}$T cells in the skin graft was further detected by immunohistochemistry. The results showed that the T cell number in the transplanted skin remarkably increased in the rejection group compared with the MSC group; Netrin-1-stimulated MSCs markedly inhibited the infiltration of T cells; compared with the rejection group, the number of T cells significantly decreased (Figure 9).

Discussion

MSCs can induce allograft immune tolerance, but low efficacy severely limits their wide application. The microenvironment may be an important factor that affects MSC function. Scientists applied MSCs in the treatment of myocardial ischemia, and the results showed that the focal hypoxia microenvironment accelerated MSC apoptosis and inhibited its pro-angiogenesis function[16]. MSCs transplanted in a diabetic ulcer were also significantly impaired, which might be attributed to the high glucose microenvironment[17]. After organ transplantation, the immune system is abnormally activated and immune cells could release a large amount of inflammatory molecules to severely damage MSCs, further promote MSC apoptosis and inhibit the exertion

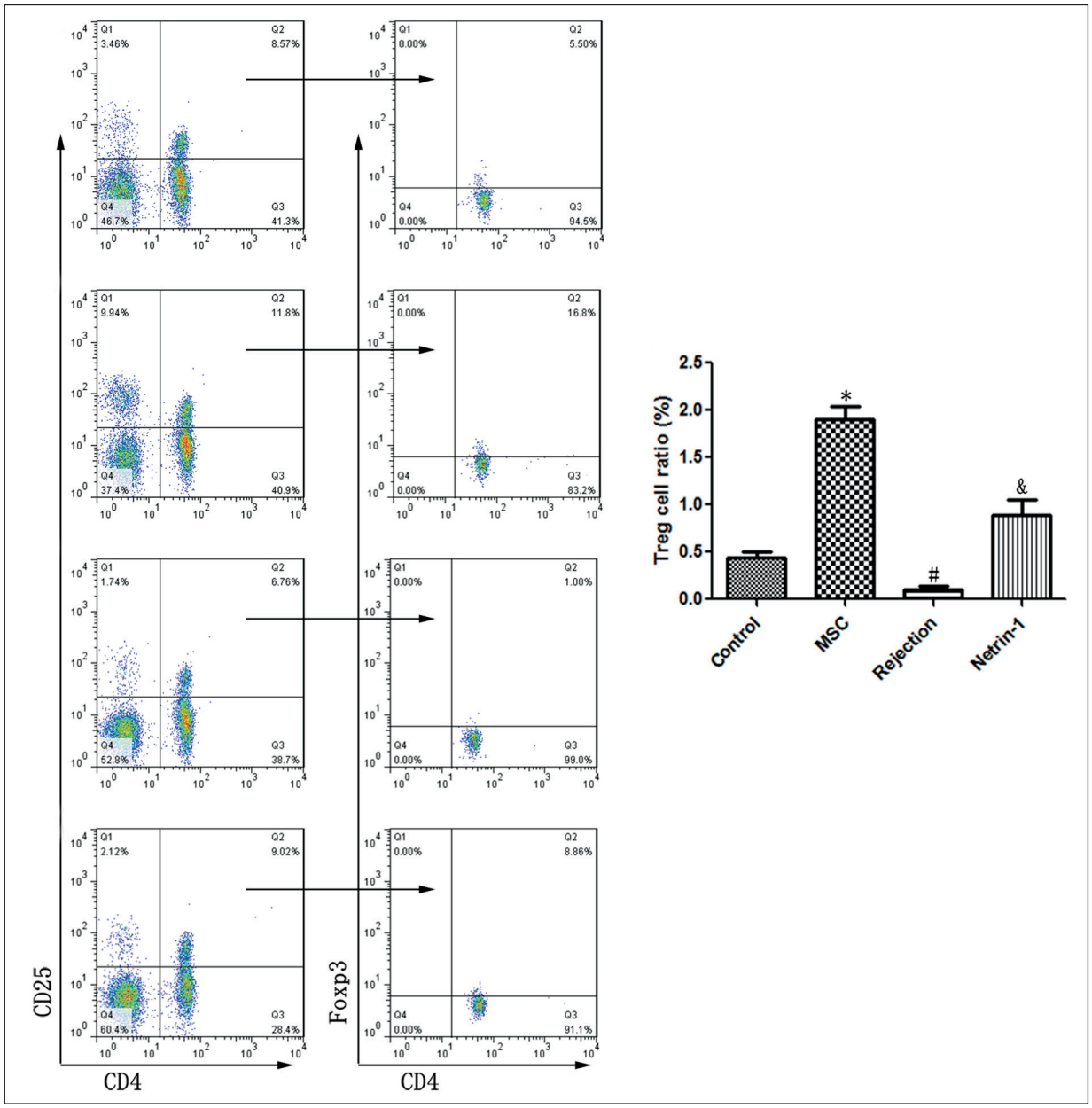

Figure 7. Netrin-1-stimulated MSC promoted the increase of Treg cell. The ratio of Treg cells in the rejection group remarkably decreased, and it decreased by 96.5% compared with the MSC group; compared with the rejection group, the ratio of Treg cells in the Netrin-1 group increased by 10.3 times. *p<0.05 (n=6) *vs.* Control; $^{\#}p$<0.05 (n=6) *vs.* MSC; $^{\&}p$<0.05 (n=6) *vs.* rejection. Values are mean ± SD.

of their function. Our study showed that MSCs in IR animals were significantly impaired, and they secreted more inflammatory molecules.

Conversely, MSC may also have two distinct phenotypes. Waterman et al[13] divided MSC into proinflammatory and immunosuppressive MSCs by TLR3 and TLR4. Svobodova et al[14] also found MSCs with different phenotypes. We believe that MSCs can be divided into immune-tolerant and pro-rejection types in organ transplantation. Subject to physiological conditions, MSCs played a strong role in inducing immune tolerance; however, when MSCs were placed in an IR microenvironment, the immune-tolerant MSC type would be transformed to the pro-rejection type, the expression of the immunosuppressive protein and the secretion of immunosuppressive molecules significantly decreased, while the se-

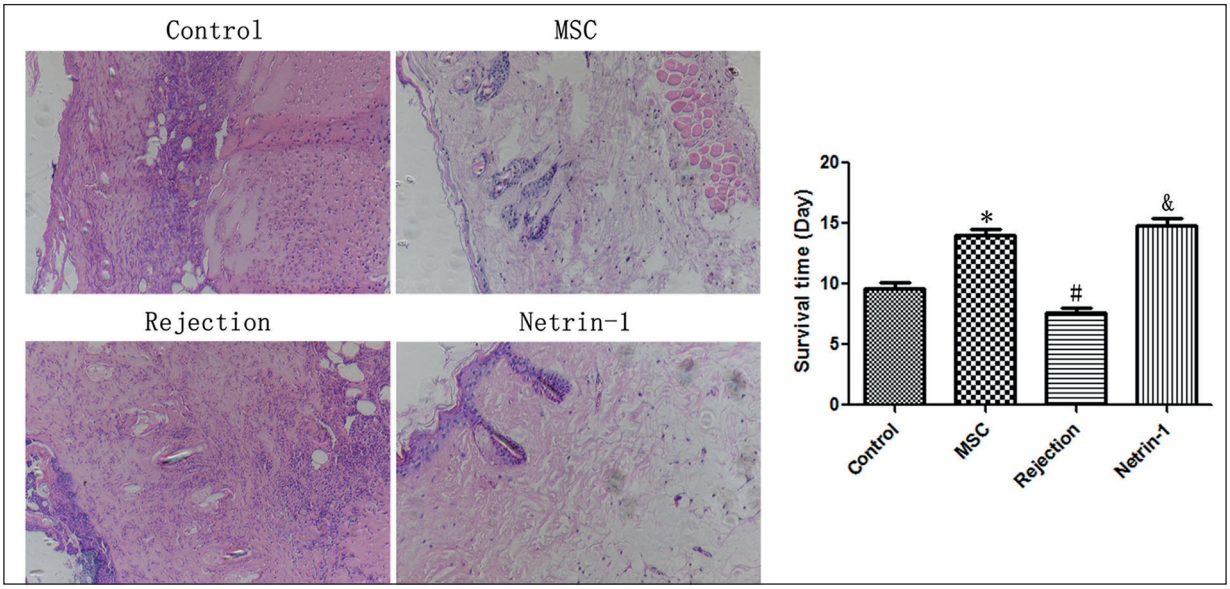

Figure 8. Netrin-1-stimulated MSC prolonged mean survival time of the skin graft (magnification: 200×). After 7 days, lymphocyte number of transplanted skin in the rejection group significantly increased; Netrin-1-simulated MSC markedly inhibited the infiltration of lymphocytes. Mean survival time of the skin graft in the rejection group was only 7.6 days, which was remarkably shorter than that in the MSC group; mean survival time in the Netrin-1 group was 14.8 days, which was significantly longer than that in the rejection group. $^{*}p<0.05$ (n=10) *vs.* Control; $^{\#}p<0.05$ (n=10) *vs.* MSC; $^{\&}p<0.05$ (n=10) *vs.* rejection. Values are mean ± SD.

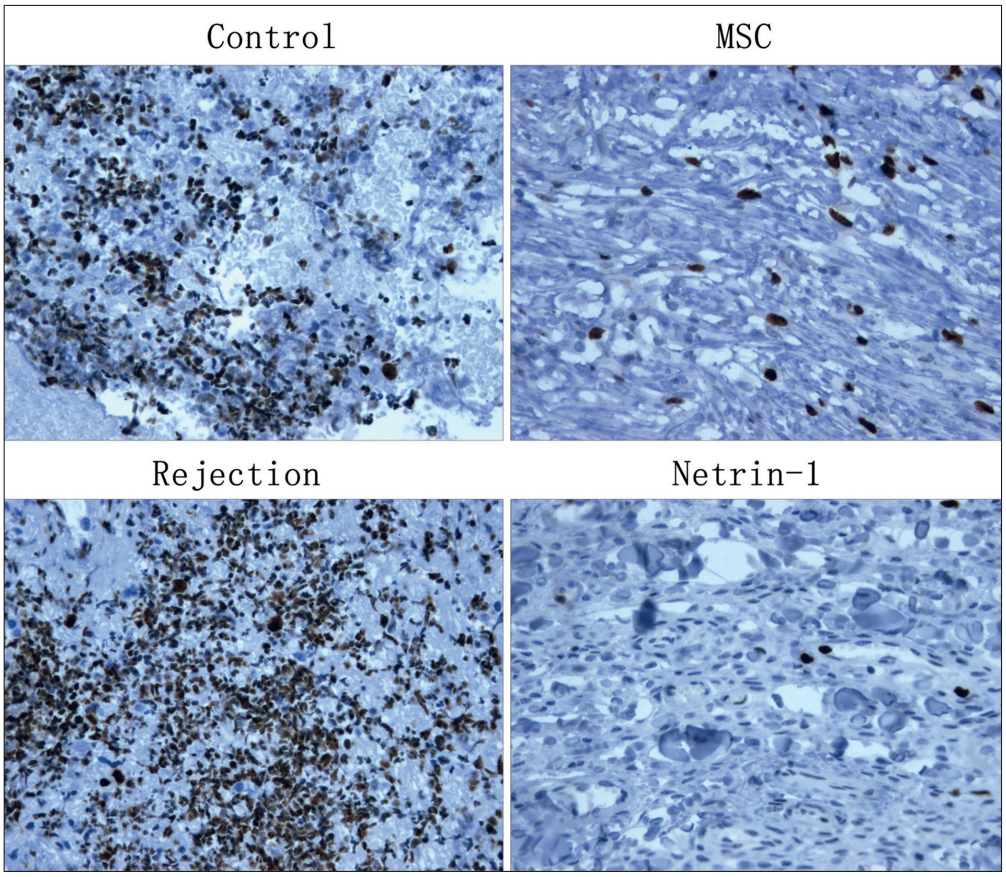

Figure 9. Netrin-1-simulated MSCs inhibited the infiltration of CD3+T cells (magnification: 200×). T cell number in transplanted skin markedly increased in the rejection group, compared with the MSC group; Netrin-1-stimulated MSC can significantly inhibit the infiltration of T cells.

cretion of proinflammatory molecules markedly increased. Netrin-1 reversed this phenotype transformation by promoting the expression of the immunosuppressive protein and the secretion of immunosuppressive molecules, ultimately facilitating the long-term survival of the skin graft. Therefore, promoting MSC transformation to the immune-tolerant type might be a promising approach for enhancing the immune tolerance of MSCs in organ transplantation.

Netrin-1 plays a crucial role in revascularization, immunoregulation, and tissue regeneration[18]. It has been reported[19,20] that Netrin-1 can maintain MSC function under ischemia conditions and facilitate the reconstruction of ischemic lower-limb blood vessels[21]. In organ transplantation, Netrin-1 can induce the polarization of the macrophage to the M2 type, inhibit the inflammatory reaction and protect the kidney against ischemia-reperfusion injury. The low expression of Netrin-1 indicated a poor prognosis for the transplanted kidney[22]. Our work found that Netrin-1 induced the transformation of MSC from the pro-rejection to immune-tolerant type and significantly extended the mean survival time of the skin graft. Thus, it is believed that Netrin-1 can serve as a target for organ transplantation. On the one hand, Netrin-1 had a cytoprotective effect that protected MSC from being damaged by inflammatory molecules[23]; on the other hand, Netrin-1 induced the transformation of MSC from the pro-rejection to immune-tolerant type and released more immunosuppressive molecules.

Conclusions

In summary, MSCs can be divided into immune-tolerant and pro-rejection types and Netrin-1 can induce the transformation of MSCs from the pro-rejection to immune-tolerant type and markedly extend the mean survival time of the skin graft.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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