Adipose-derived mesenchymal stem cells suppress of acute rejection in small bowel transplantation

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Abstract

Background/Aims: Adipose-derived mesenchymal stem cells (ADSCs) possess immunosuppressive activity and hold promise in autologous cell-based therapies. The aim of this study was to determine whether autologous ADSCs can improve outcomes in the rat small bowel transplantation (SBT) model.

Materials and Methods: Allogeneic SBT followed by implantation of autologous ADSCs through the penile vein was conducted in Brown-Norway (BN) donor rats with Lewis (LEW) recipient rats infused with phosphate buffered solution as the control. Allograft and recipient peripheral blood were obtained. We assessed histopathology, apoptosis, cytokines, and regulatory T cells (Tregs). One-way analysis of variance was applied to assess the significance of the data.

Results: It was found that ADSCs significantly reduced acute rejection and improved the allograft's survival rate. In addition, there were significantly fewer apoptotic cells in allograft mucosae in the ADSC group in comparison with the control group. Furthermore, levels of interleukin (IL)-10 and transforming growth factor-β1 were significantly elevated, whereas those of IL-2 and IL-17 levels were significantly reduced in the ADSC group when compared to the control group. Moreover, flow cytometry analysis revealed that there were significantly more peripheral Tregs after the infusion of ADSCs.

Conclusions: These results demonstrate that implanted autologous ADSCs improve allogeneic small bowel allograft outcomes by attenuating the acute rejection and reducing inflammatory responses.

Keywords: Acute rejection, adipose-derived mesenchymal stem cells, small bowel transplantation

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INTRODUCTION

Small bowel transplantation (SBT) is now a useful therapeutic options for patients with irreversible intestinal failure.[1] In particular, for patients who have developed life-threatening complications related to long-term parenteral nutrition, SBT is the only life-saving treatment.[2] Since the first successful clinical SBT conducted by Deltz *et al.* (1989),[3] over 5000 adult and pediatric SBTs have been performed around the world.[4] In the early experience with

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SBT, acute rejection was an almost universal complication due to the highly immunogenic features of the transplant.[5] Unfortunately, long-term administration of conventional immunosuppressive agents to control rejection increases the risks of unfavorable side effects.[6] Therefore, new immunosuppressive strategies to combat rejection after SBT are urgently needed.

A number of previous studies have shown that adipose-derived mesenchymal stem cells (ADSCs) are a readily accessible, abundant, and reliable stem cell source for stem cell-based therapy, not only because of their antiinflammatory and immunomodulatory functions capable of reducing the risk of graft rejection[7,8] but also due to their propensity for self-renewal and multidifferentiation potency. It has been speculated that ADSCs might also be the optimal choice for autologous cell transplantation.[9] Our previous study demonstrated that autologous transplantation of ADSCs markedly alleviated allograft rejection of renal transplantation through the suppression of inflammation, apoptosis, and immune over-reactivity.[10] As far as we are aware, there have been no publications investigating whether implanted autologous ADSCs can improve outcomes of allogeneic SBT.

Based on the above observations, we performed autologous ADSCs transplantation during experimental SBT and examined whether the implanted ADSCs could decelerate graft rejection, and thus prolong the postoperative survival days of recipient rats.

MATERIALS AND METHODS

Animals
Adult male Brown Norway (BN) rats weighing 220–250 g and adult male Lewis (LEW) rats weighing 220–250 g were obtained from the Academy of Military Medical Science [Beijing, People's Republic of China; certificate no. SCXK (JUN) 2007-004]. Animal procedures were approved by the Ethics Committee of Tianjin First Central Hospital (Tianjin, People's Republic of China). Rats were bred and maintained under specific pathogen-free conditions with free activity and free access to water and rodent chow. Ambient temperature was controlled at 22–24°C under a 12 h light/dark cycle. The rats were fasted for 12 h prior to surgery and provided with free access to 10% glucose water following surgery. All of the surgical procedures were performed under sanitary conditions; all the experiments were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals.[11] The present study was approved by the Ethics Committee of Tianjin First Central Hospital (Tianjin, People's Republic of China).

Adipose-derived mesenchymal stem cells preparation
ADSCs were isolated and cultured from LEW rats by the method described earlier.[12] Briefly, aseptically excised and minced inguinal adipose tissue was digested enzymatically with collagenase type I solution (Sigma-Aldrich, St. Louis, MO, USA) for 60 min at 37°C with constant agitation (100 rpm). After centrifugation, the stromal cell fraction was filtered and cultured in Dulbecco's Modified Eagle's medium (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco) and antibiotics. When the cells reached confluence, the adherent cells were detached with trypsin/ethylenediamine tetraacetic acid (Sigma-Aldrich) and re-seeded for expansion. Fourteen day(s) *in vitro* culture at 37°C, 5% CO_2, and 95% humidity were needed in order to obtain enough ADSCs for the autologous transplantation. The cultured ADSCs (3×10^6) from each experimental rat were labeled and cryopreserved in liquid nitrogen (Tianjin AIR Products Co., Tianjin, People's Republic of China) prior to injection.

The cultured ADSCs were identified via the expression of hematopoietic marker, CD34 and CD45, and mesenchymal cell markers, CD90, CD73, and CD105 using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA), and data were analyzed using the CellQuest software program. The ADSCs which had differentiated into adipocytes, islet-like cells, and osteoblasts *in vitro* were also tested using our earlier described method.[13]

Surgical procedures and experimental protocol
An experimental SBT model was performed with BN donor rats and LEW recipient rats in a sterile field under general anesthesia with isoflurane inhalation (LUNAN Pharmaceutical Co., Ltd., Shandong, People's Republic of China) administered via an isoflurane vaporizer (Matrx VMR, MIDMARK Corporation, OH, USA). There was <10 g weight difference between the donors and the recipients. Allogeneic heterotopic SBT was performed using standard microvascular techniques as described earlier.[14] All surgical procedures were performed by a single operator under the naked eye. Immediately after surgery and graft reperfusion, rats were divided into two groups. The ADSC group was injected with 2.0×10^6 autologous ADSCs suspended in 1 ml phosphate buffered solution (PBS) through the penile vein, followed by an injection at 6 h after the reperfusion procedure again via the penile vein. The control group received the equivalent volume of PBS. After the procedure, the animals were resuscitated, heated with thermal blankets, and placed in

individual cages. Five animals per group were euthanized as described earlier on postoperative day (POD) 7. We collected the allografts and blood samples. A portion of the tissue was fixed, with the remaining tissues and serum samples stored at −80°C. There were five animals per group in the survival analysis.

Survival rates of recipients
Recipient survival rates were examined in five animals per group. Humane endpoints were used for moribund animals after surgery, especially in the survival study. All animals meeting the humane endpoint criteria were euthanized by deep anesthesia as described earlier.

Histopathological analysis
Grafts were fixed overnight in formalin, dehydrated and then embedded in paraffin wax, and cut into 5-μm thick sections. In the histological evaluation, sections were deparaffinized and rehydrated and then were stained with hematoxylin and eosin (H and E; Sigma-Aldrich) following standard methods for routine morphological analysis. Each slide was viewed in a Nikon microscope (Ni-U, Nikon Corp., Tokyo, Japan). Acute rejection was classified using the Wu score.[15]

Apoptosis assay
Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end-labeling (TUNEL) staining was conducted on paraffin-embedded tissue sections using the *In Situ* Cell Death Detection Kit (Roche Biochemicals, Mannheim, Germany) according to the manufacturer's guidelines. In this method, apoptotic nuclei appear brown, while the staining of the cytoplasm is generally light or absent.[16]

Enzyme-linked immunosorbent assay
Serum interleukin (IL)-2, IL-10, transforming growth factor (TGF)-β1, and IL-17 concentrations were measured with enzyme-linked immunosorbent assay kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

Analysis of CD4$^+$ CD25$^+$ Foxp3$^+$ T cells in peripheral blood by flow cytometry
The peripheral blood mononuclear cells were stained with anti-CD4-fluorescein isothiocyanate (cat no. 11-0040), anti-CD25-phycoerythrin (cat no. 12-0390), and anti-Foxp3-PerCP-Cyanine5.5 (cat no. 45-5773) antibodies (eBioscience, San Diego, CA, USA). Isotype-matched control antibodies served as staining controls. A FACSCalibur flow cytometer (BD) was utilized to determine CD4$^+$ CD25$^+$ Foxp3$^+$ T cells (Tregs) and data analysis was performed using CellQuest software (BD Biosciences).

Statistical analysis
Data from repeated experiments are presented as the means ± standard deviation. The results were analyzed by one-way analysis of variance. In the survival analysis, the Kaplan–Meier method was used. The log-rank test was employed to check for equality of the survival distributions. Statistical analyses were performed using Statistical Package for the Social Sciences version 16.0 software (SPSS Inc., Chicago, IL, USA). In all evaluations, $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of adipose-derived mesenchymal stem cells
The adherent ADSCs had a spindle-shaped fibroblastic morphology after expansion. The ADSCs of rat expressed typical markers and differentiation profiles, i.e., in the flow cytometry analysis, they displayed intense expression of the stem cell markers, CD90, CD73, and CD105, but were negative for the hematopoietic markers, CD34 and CD45 [Figure 1a]. In addition, culture-expanded ADSCs were also functionally capable of differentiating into adipocytes, islet-like cells, and osteoblasts under inductive culture conditions; this was confirmed with Oil Red O, dithizone, and Alizarin red S staining [Figure 1b].

Improvements in recipient survival rates
The median recipient survival times were 18 days in the ADSC group, which was 7 days longer that the corresponding value in the control group (11 days). Statistical analysis of the survival days by log-rank test revealed a significant difference between control and ADSC group rats ($n = 5$; $P < 0.01$; [Figure 2]). These results indicated that ADSC therapy had improved the survival of the recipients.

Histopathological analysis and grading of acute rejection in grafts
Allogenically transplanted animals treated with PBS displayed signs of moderate or severe acute rejection when assessed at POD 7. The pathological examination revealed many changes, i.e., elevated infiltration by inflammatory cells both in the mucosa and submucosa, crypt epithelial injury, a higher abundance of crypt apoptotic bodies, architectural distortion as well as mucosal ulceration and hemorrhage. In contrast, the pathology of the ADSC group was characterized by mild rejection at POD 7 [Figure 3]. The rejection index was significantly lower on POD 7 postoperatively compared with the control group ($n = 5$; $P < 0.01$; [Figure 4]).

Allograft apoptosis
Amelioration of tissue damage could be demonstrated by the reduced number of apoptotic cells as confirmed using
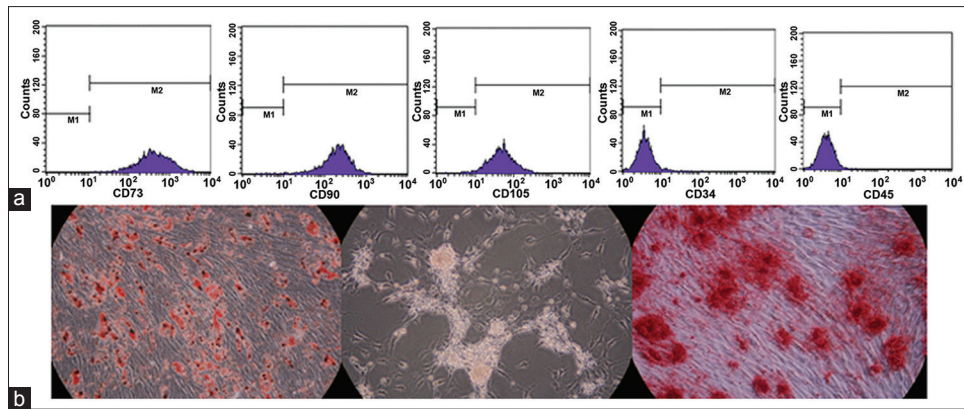
Figure 1: Characterization of adipose-derived mesenchymal stem cells (ADSCs). (a) Flow cytometric characterization of ADSCs. ADSCs intensely expressed CD73, CD90 and CD105, but did not express CD34 and CD45. (b) The ADSCs were induced to differentiate into adipocytes (left panel), islet-like cells (middle panel), or osteoblasts (right panel) ×200

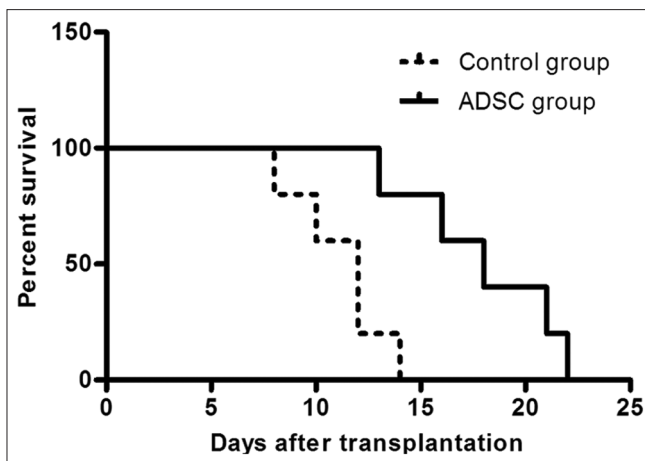**Figure 2:** Kaplan-Meier curve for survival times in the allogenic small bowel transplantation model
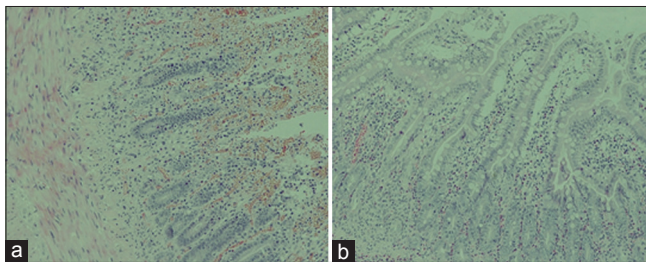

Figure 3: The histopathology of intestinal specimens collected on days 7 post-transplantation with and without ADSCs treatment. Representative photomicrographs are shown ×200. (a) The control group on POD 7 and (b) the ADSC group on POD 7

TUNEL staining in the ADSC group when compared with the control group ($n = 5$; $P < 0.01$; [Figure 5]).

Recipient serum cytokine concentrations

The serum expression levels of IL-2 and IL-17, i.e., proinflammatory cytokines, were significantly higher in the control group than in the ADSC group ($n = 5$; $P < 0.01$; [Figure 6]). The situation was reversed with respect to the expression levels of IL-10 and TGF-β1, which are indicators of antiinflammation/immunomodulation; their concentrations were notably lower in the control group than in their ADSC counterparts ($n = 5$; $P < 0.01$; [Figure 7]). These findings clearly suggested that infusion of ADSCs had inhibited the inflammatory reaction in this experimental setting.

Effect of ADSCs therapy on Treg numbers in peripheral blood

Flow cytometry was used to estimate the number of Tregs in the peripheral blood after SBT. The proportion of Tregs in the ADSC group was significantly higher than the corresponding value in the control group on POD 7 ($n = 5$; $P < 0.01$; [Figure 8]).

DISCUSSION

SBT is now being performed in clinical practice as it is one of the most promising therapies for irreversible intestinal failure.[17] In this study, the immunosuppressive potential of autologous ADSCs in the treatment of acute rejection after allogenic SBT was demonstrated both histologically and functionally.

Mesenchymal stem cells (MSCs) are multipotent mesenchymal stromal cells that can be obtained from bone marrow, umbilical cord blood, and adipose tissue.[18] Recent studies in a rat model have demonstrated that MSCs isolated from bone marrow were able to ameliorate a small bowel ischemia/reperfusion injury.[19,20] Qu *et al*. examined this property in a rat model of inflammatory bowel disease; they demonstrated that *in vitro* expanded MSCs isolated from bone marrow relocate to the injured intestinal tissues where they can undergo local proliferation.[21] They also appear to transdifferentiate into intestinal mucosal cells during the first few weeks

posttransplantation. Therapy with MSCs is associated with improved mucosal regeneration in indomethacin-injured rat intestine. Adipose tissue is an abundant source of MSCs. In the study of Linero and Chaparro,[22] it was demonstrated that ADSCs promoted bone regeneration not only due to their potential to differentiate and integrate into the injured tissue but also to their ability to secrete soluble factors that facilitated the endogenous process of regeneration. Because of ready accessibility and good expansion properties, we decided to evaluate the therapeutic activity of ADSCs in SBT.
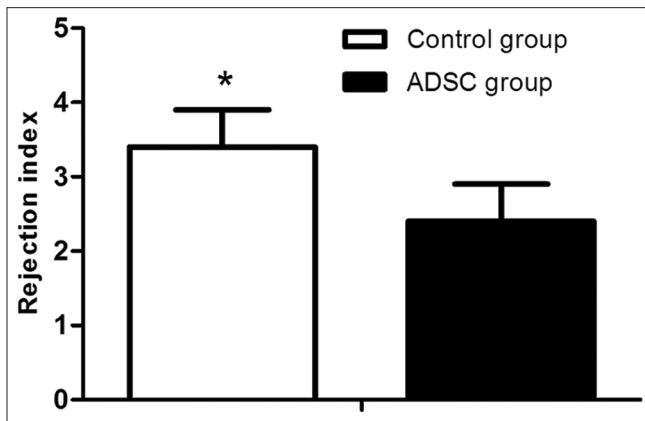

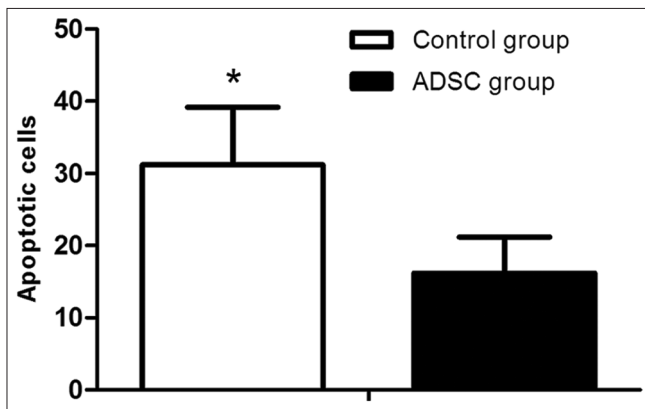**Figure 4:** The assessment of the rejection index. *P < 0.01 vs. ADSC group

Figure 5: The apoptotic cells in graft mucosae. *P < 0.01 vs. ADSC group

In the current study, an experimental SBT model was established with major histocompatibility complex-disparate rat strains (BN to LEW).[14] Pathological analysis revealed a moderate-to-severe allograft acute rejection in the control group, including an aggravated inflammatory cell infiltration both in the mucosa and submucosa, crypt epithelial injury, a higher abundance of crypt apoptotic bodies, architectural distortion as well as mucosal ulceration and hemorrhage.[23] The histologic grading of acute rejection clearly revealed that the ADSCs treated animals suffered from a less severe acute rejection than the untreated controls at POD 7. The graft mucosal architecture was better preserved and crypt epithelial injury was less severe after administration of ADSCs. Furthermore, therapy with ADSCs clearly prolonged the survival of the recipients. Therefore, these results confirmed the hypothesis that ADSCs confer a prominent allograft protective benefit.

Furthermore, it is now evident that epithelial apoptosis is the mechanism of cell death in allograft rejection.[24] In the present study, less extensive cell apoptosis was found in the allograft after autologous ADSCs transplantation. Du *et al*.[25] observed fewer apoptotic cells after MSC-conditioned medium infusion in transplantation models. Here, the reduction in the extent of epithelial apoptosis after ADSCs therapy may, at least in part, be attributed to a reduction in the levels of proinflammatory cytokines in the bloodstream, i.e., fewer Th1 cells producing IL-2, and Th17 cells secreting IL-17, simultaneously accompanied by increases in the numbers of Th2 cells releasing the antiinflammatory cytokine IL-10 and Th3 cells with TGF-β1 expression. Yang *et al*.[26] reported that Th17 cells and IL-17 might play an important role in human and rat small bowel acute transplantation rejection. The administration of anti-IL-17 mAb could significantly suppress the acute rejection degree of rat intestine grafts and prolong the survival time of recipient rats. This suggests that the ADSCs may have a role in regulating the inflammatory response and play a protective role by changing the balance between the levels of inflammatory and antiinflammatory cytokines circulating in response to the SBT.
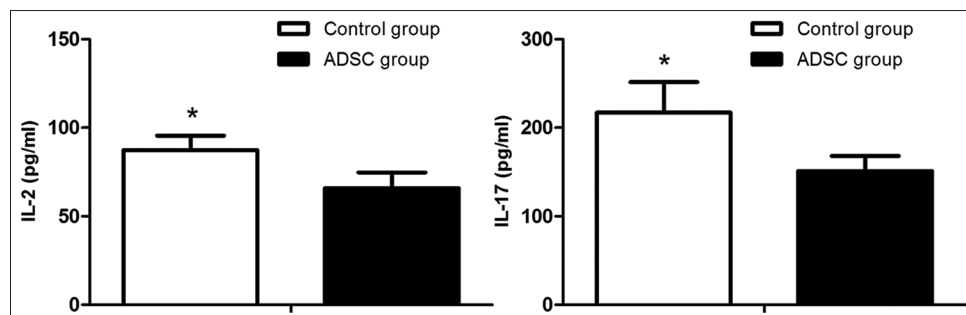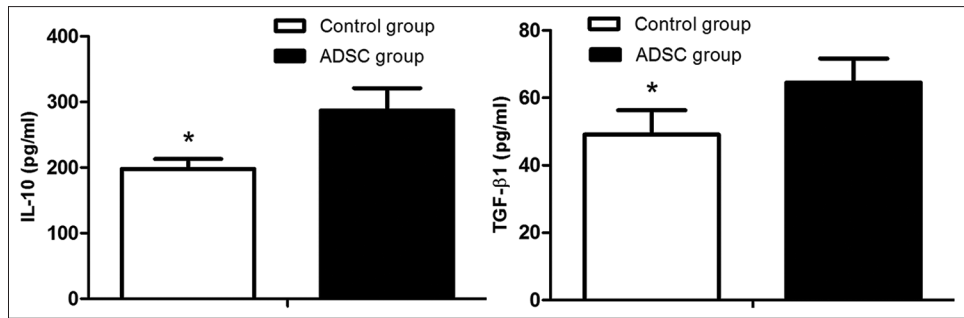

Figure 6: Th1 and Th17-associated cytokine concentrations in serum of the transplant recipients. *P < 0.01 vs. ADSC group

Figure 7: Th2, and Th3-associated cytokine concentrations in serum of the transplant recipients. *$P < 0.01$ vs. ADSC group
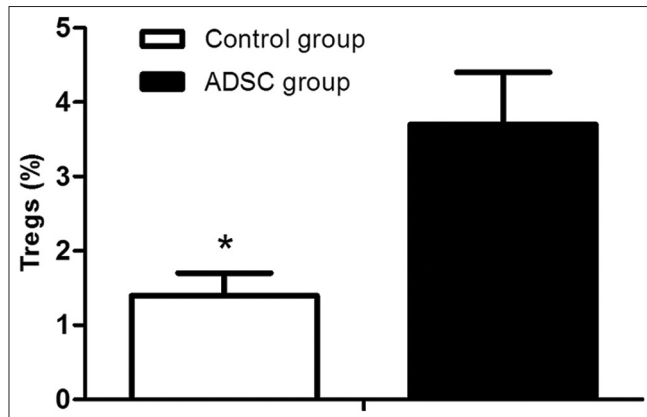

Figure 8: Proportion of Tregs in recipient peripheral blood. CD4+ CD25+ Foxp3+ T cells (Tregs) in peripheral blood. *$P < 0.01$ vs. ADSC group

Tregs account for approximately 5 to 10% of peripheral CD4+ T cells; these cells exert negative immune regulatory effects. By inhibiting the activation and proliferation of other immune effector cells, Tregs play an important role in the induction of immune tolerance to transplanted tissue.[27] In a rat SBT model of acute rejection, Yang *et al*.[28] reported that the percentage of Tregs decreased in the transplant. Donor-specific Treg cells acquired from tolerant mice after *in vitro* expansion generated a stable chimerism promoting acceptance of the intestinal allograft and it has been claimed that increased numbers of intragraft Treg cells and clonal deletion contribute to the development of SBT tolerance.[29] At present, the following mechanisms have been implicated in the Treg-mediated inhibition of other immune effector cells: inhibiting IL-2 production due to close contacts between cells; not allowing antigen presenting cells to express stimulus molecules and preventing dendritic cells from maturing; destroying target cells via the granular enzyme/perforin death pathways; consuming local IL-2 leading to a loss of stimulatory signals to effector T cells; and inducing the production of inhibitory cytokines such as TGF-β1 and IL-10.[30] In our study, the proportion of Tregs was significantly higher in the ADSC group than in the controls on POD 7. Therefore, we conclude that one mechanism by which ADSCs can

exert their immunosuppressive effects is by inducing the production of Tregs.

In conclusion, our study reveals that autologous ADSC therapy after SBT attenuates acute rejection, and inhibits apoptosis, all of which contribute to improving recipient survival.

CONCLUSION

We found that autologous ADSC prolonged the life span of recipient through attenuating the acute rejection and reducing inflammatory responses.

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Conflicts of interest

There are no conflicts of interest.

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