Allogeneic mesenchymal stem cell treatment alleviates experimental and clinical Sjögren’s syndrome

Running head: ALLOGENEIC MSCs TREATMENT FOR SS

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Abstract

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by dry mouth and eyes, and the cellular and molecular mechanisms for its pathogenesis are complex. Here, we revealed for the first time that bone marrow mesenchymal stem cells (BMMSCs) in SS-like NOD/Ltj mice and human patients were defective in immunoregulatory functions. Importantly treatment with mesenchymal stem cells (MSCs) suppressed autoimmunity and restored salivary gland secretory function in both mouse models and Sjögren's syndrome patients. MSC treatment directed T cells towards Treg and Th2, while suppressing Th17 and Tfh responses, and alleviated disease symptoms. Infused MSCs migrated toward the inflammatory regions in a stromal cell-derived factor-1 (SDF-1) dependent manner, as neutralization of SDF-1 ligand CXCR4 abolished the effectiveness of BMMSC treatment. Collectively, our study suggests that immunological regulatory functions of MSCs play an important role in SS pathogenesis, and allogeneic MSC treatment may provide a novel, effective, and safe therapy for patients with SS. This study is registered at ClinicalTrials.gov (phase I/II, Identifier: NCT00953485).
Introduction

Human Sjögren’s syndrome (SS) is a chronic, systemic autoimmune disorder characterized by inflammation of exocrine glands and functional impairment of the salivary and lacrimal glands. B cells have been shown to play a significant role in SS pathogenesis, as their depletion significantly alleviated disease symptoms. Remarkable reduction of Treg numbers in salivary glands and reduction of CD4⁺CD25⁺high T cells in peripheral blood were observed, and analysis of inflammatory tissue in the salivary glands showed a predominance of T cells, in particular Th1 cells infiltration, albeit Th2 and Th17 responses have been also reported, demonstrating the complexity of the SS pathogenesis. Moreover, because methods for salivary gland morphological (sialogram) observation and functional (saliva flow rate) evaluation are both non-invasive and easy to operate, SS serves as a valuable model for autoimmune disease studies.

Mesenchymal stem cells (MSCs), such as bone marrow mesenchymal stem cells (BMMSCs) and umbilical cord mesenchymal stem cells (UCMSCs), are multipotent stem cells with the capacity to differentiate into osteoblasts, chondrocytes, adipocytes, and neural cells. MSCs express low levels of MHC class I but lack expression of MHC class II surface molecules, and thereby cannot serve as effective antigen-presenting cells to promote immune responses. Although the precise molecular mechanism remains unclear, MSCs have been reported to exert immunomodulatory effects on various activated lymphoid cells including T cells, B cells, natural killer cells, and dendritic cells. Their low immunogenicity and
immunoregulatory potential offer a promising new treatment for severe refractory autoimmune diseases.\textsuperscript{14-19} Indeed, the therapeutic efficacy of MSC infusion has been demonstrated in experimental and clinical systemic lupus erythematosus,\textsuperscript{20,21} systemic sclerosis\textsuperscript{22} and type 1 diabetes mellitus.\textsuperscript{23} In addition, migrating MSCs may also represent a source of multipotent cells that could repair damaged tissues and organs. The underlying mechanisms responsible for the homing of infused MSCs remain unclear.

Currently, treatment of SS is difficult and challenging.\textsuperscript{24} For example, in contrast to other inflammatory autoimmune diseases including rheumatoid arthritis, blocking TNF-\(\alpha\) showed very little effect in treatment of SS.\textsuperscript{7} Since MSCs offer a promising new treatment for autoimmune diseases, we examined functions of MSCs in SS disease mouse models and human SS patients, determined whether allogeneic MSCs have therapeutic effects and investigated the underlying mechanisms of MSC treatment in both experimental animal models and SS patients.

**Materials and methods**

**Mice**

Female NOD/Ltj mice (\textit{Cdh23ahl}) served as SS animal model, with the \textit{H}-2 haplotype \textit{H}-2\textsuperscript{g7}; outbred strain ICR mice (from a sub-line of which the NOD strain were derived) served as control. Male BALB/c mice (\textit{H}-2\textsuperscript{d}) and C57BL/6-gfp (\textit{H}-2\textsuperscript{b}) transgenic mice served as allogeneic MSC donors. NOD/Ltj mice and ICR mice were purchased from Beijing HFK Bioscience Co., LTD, China, BALB/c mice and
C57BL/6-gfp transgenic mice were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. Mice were maintained in a specific pathogen-free animal facility and kept under conventional conditions with free access to water and food. The Animal Care and Use Committee of Capital Medical University approved all experiments in this study.

**Isolation, culture, differentiation, and CXCR4 blockade of BMMSCs *in vitro***

Bone marrow cells were flushed out from bone cavity of femurs and tibias with heat-inactivated medium. After 3-4 passage, BMMSCs were cultured under osteogenic or adipo-induction differentiation condition for 2 weeks, and the osteogenic and adipogenic differentiation potentials were determined. Neutralizing anti-CXCR4 antibody (5μg/ml/5 × 10⁵ cells, R&D Systems) was added and incubated in 37°C for 20 min. Then cells were harvested for infusion. Details were described in supplemental methods.

**T cell proliferation**

After isolation, splenocytes and peripheral blood mononuclear cells (PBMCs) were labeled by Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) stimulated by anti-CD3 antibody, with or without BMMSCs from SS disease animals, normal animals, SS patients or normal human beings respectively. After 4 days, lymphocytes were harvested and determined by flow cytometric analysis. Proliferation index was
calculated, as the average number of cell divisions versus the original population by Modfit LT 3.0 software. Details were described in supplemental methods.

**Allogeneic BMMSC treatment and saliva flow rate measurement**

For BMMSC treatment, NOD/Ltj mice were injected with BMMSCs (from BALB/c or C57BL/6-gfp; \(1 \times 10^5\) cells/mouse) in 0.15 ml PBS via tail vein. Saliva flow rate was determined as described in supplemental methods.

**Histological analysis of salivary glands**

Samples were fixed with 4% PFA for 24 hours at 4 °C; paraffin sections were used for H&E staining and frozen sections were used for GFP-BMMSC tracking analysis. After H&E staining, the sections were photographed and area of inflammatory focus (containing >50 lymphocytes per 4 mm² tissue) were calculated per field at \(\times200\) magnification by Image-Pro Plus version 6.0 software. Five entire salivary gland sections for each animal were counted with an average of 10 fields/gland by an experienced expert of histopathology under blinded fashion.

**Real-time RT-PCR**

Total RNA was isolated by RNA isolation kit (Sunbio, China) following the manufactures’ instruction, and cDNA was synthesized from 100 ng of total RNA in 3 μl using reverse transcription kit (Sunbio, China). Real-time PCR, was analyzed using the delta-delta-CT method. A detailed description of procedures and primers specific
for gene regions is provided in supplemental methods.

**ELISA**

Mice peripheral blood was collected from the retro-orbital plexus of treated mice and controls and centrifuged to obtain serum. Tissue lysates were extracted from mouse spleen, liver, lung, kidney, salivary gland and lymph node. Bone marrow cells were flushed out from bone cavity of femurs and tibias with PBS. For human, serum samples were collected before UCMSC treatment and at each follow-up visit. Cytokines were assessed by mouse or human ELISA kit (R&D Systems) according to the manufacture’s instruction.

**Flow cytometric analysis**

Flow cytometric analysis was performed as previously described.25 Mouse splenocytes were used for cytometric analysis in the animal studies. Detail methods for Treg, Tfh, cytokine-producing cells (Th1, Th2, Th17 cells) staining are provided in supplemental methods.

**Patient enrollment**

Twenty four primary SS patients ranging in age from 27 to 68 years, were enrolled in an MSC treatment trial. Inclusion and exclusion criteria are reported in supplemental methods. All patients enrolled in the present study were poor responsive to glucocorticoid/glucocorticoid combined with immunosuppressant treatment before
MSCT. Eleven patients had severe xerostomia and/or xerophthalmia at baseline, while the other 13 patients had refractory systemic comorbidities, including thrombocytopenia in 4 patients, anaemia in 3 patients, interstitial lung disease in 2 patients, renal tubule acidosis (RTA) in 4 patients, autoimmune hepatitis in 7 patients, nervous system involvements in 3 patients, autoimmune enteritis in 1 patient. The trial was conducted in compliance with current Good Clinical Practice standards and in accordance with the principles set forth under the Declaration of Helsinki, 1989. This protocol was approved by the Ethics Committee at The Drum Tower Hospital of Nanjing University Medical School and registered at ClinicalTrials.gov (phase I/II, Identifier: NCT00953485). All patients provided informed consent.

Umbilical cord mesenchymal stem cell infusion

UCMSCs were prepared by the Stem Cell Center of Jiangsu Province; and details of UCMSCs purification and identification were described previously21 and in supplemental methods. For treatment, UCMSCs (1 × 10⁶/kg of body weight) were administered by intravenous infusion without premedication such as steroids or antihistamines.

Follow-up procedures

After mesenchymal stem cell treatment (MSCT), all 24 patients were followed and data collected at baseline, 2 weeks, 1, 3, 6, and 12 months after MSCT. The SS Disease Activity Index (SSDAI) score²⁶ and visual analog scale (VAS) for assessment
of disease impact were assessed and blood samples were taken for laboratory examinations including hemoglobin, alanine aminotransferase and aspartate aminotransferase for each patient at each follow up visit. For those with severe xerostomia and/or xerophthalmia at baseline, assessments performed at these time points included salivary flow rate test, determination of modified treatment emergent symptom scale (TESS) for xerostomia score\textsuperscript{27} and sialography. Systemic improvements were graded by VAS score and SSDAI score at each visit time for those without obvious xerostomia and/or xerophthalmia at baseline. Adverse events and their severity were assessed and recorded throughout the study. Sialography of the parotid gland\textsuperscript{28,29}, saliva flow rate measurements, VAS score and SSDAI determination for SS patients are given in the supplemental methods. Medication intake was tapered according to disease amelioration.

**Statistical analysis**

All data have a normal distribution and presented as means ± standard error (s.e.m. in data of in infiltrating area statistics) or standard deviation (s.d. in other data) of three independent experiments and we used an alpha level of 0.05 for all statistical tests. The mice salivary flow rate and all clinical trial data were statistically analyzed with repeated measurement; other data were analyzed with one-way analysis of variance (ANOVA) using SPSS 17.0. Each value was compared with the control values. $P < 0.05$ was regarded as statistically significant and was adjusted by the Bonferroni method to allow for multiple comparisons. The chart was made by Microsoft Office.
Results

Immunoregulatory activities of BMMSCs are impaired in disease mice and SS patients

Previous studies suggest that T lymphocyte over-activation is associated with functional impairment of BMMSCs.\textsuperscript{30} We first examined the immunoregulatory activity of BMMSCs in a nonobese diabetic mouse model (NOD/Ltj). Splenic cells from ICR mice were cultured with ICR or NOD/Ltj BMMSCs, plus anti-CD3 and anti-CD28 mAbs. As shown in Figures 1A and B, culturing T cells with NOD/Ltj BMMSCs resulted in much higher proliferative responses compared to culturing with ICR BMMSCs, indicating that immunoregulatory activities of BMMSCs were reduced in disease animals. Interestingly, we observed that the frequency of CD4$^+$Foxp3$^+$ T regulatory cell (Treg) was significantly lower ($P=0.0001$, n=12; Figure 1C, E) in T cells cultured with BMMSCs from disease animals, and the percentage of CD4$^+$ cells in splenocytes co-cultured with NOD/Ltj BMMSCs was significantly higher than that with ICR BMMSCs ($P=0.0005$, n=12; Figure 1C, D), suggesting a possible mechanism that reduced Treg development driven by BMMSC from disease animals contributed to their loss of immunosuppression activities. BMMSCs from NOD/Ltj mice have significantly lower proliferative capacity ($P=1.691 \times 10^{-11}$, n=6; Figure S1A, B), and less osteogenic (Figure S1C-E) and adipogenic (Figure S1F-H) differentiation potentials than those from ICR mice,
indicating that other biological functions of BMMSCs are also impaired. To determine if the impairment of immunoregulatory activities we observed in disease animals also exist in SS patients, normal human peripheral blood mononuclear cells (PBMCs) were cultured with BMMSCs obtained from normal persons or SS patients. As shown in Figures 1F and 1G, BMMSCs from SS patients showed significant defectiveness of immunoregulatory functions, as much higher proliferative responses were observed in cells co-cultured with BMMSCs obtained from SS persons than from normal patients \( (P =0.01, n=4) \). Together, these results demonstrated that the loss of immunoregulatory functions by BMMSCs may play an important role in SS pathogenesis.

Treatment of normal allogeneic BMMSCs alleviates SS-like disease in NOD/Ltj mice

SS-like autoimmune disorders usually appear at age 7-8 weeks in NOD/Ltj mice. To investigate whether BMMSCs have preventive and therapeutic effects on SS-like inflammation in mice, we infused allogeneic BMMSCs from BALB/c mice into NOD/Ltj mice either at an early stage of SS (6 weeks of age, prevention group) or at the developed stage (16 weeks of age, treatment group). In NOD/Ltj mice, the inflammatory responses in the submandibular glands increased rapidly from 6 to 8 weeks of age \( (P =0.00002, n=6; \text{Figure 2A, B}) \). When NOD/Ltj mice were injected with allogeneic BMMSCs at 6 weeks of age, the area of inflammation in the submandibular glands was significantly smaller than untreated NOD/Ltj mice \( (P \)
=0.017, n=6), and this protective effect was detectable as early as 2 weeks after BMMSCs infusion (Figure 2A, B). A similar reduction of inflammatory responses was observed in treatment group (BMMSCs were infused at 16 weeks of age) when compared with untreated NOD/Ltj mice (P =0.009, n=6; Figure 2A, C). The saliva flow rate of untreated NOD/Ltj mice began to decrease at the age of 6 weeks, and declined rapidly thereafter (P =0.008, 8 weeks versus 4 weeks of age, n=6; Figure 2D). In the prevention group, mice that received BMMSCs infusion at 6 weeks of age showed sustained saliva flow rate 2 weeks after BMMSC infusion (P =0.977, 8 weeks of age versus 4 weeks of age, n=6; Figure 2D), and the saliva flow rate also improved significantly in the treatment group 2 weeks after BMMSCs infusion (P =0.045, 18 weeks versus 16 weeks of age, n=6; Figure 2E). These findings indicated that allogeneic BMMSCs were effective in suppressing inflammation and restoring secretory function of the salivary glands in SS-like disease mice.

**SDF-1/CXCR4 axis is critical for MSCs to migrate and improve salivary gland function**

We next investigated the underlying mechanisms by which the injected allogeneic BMMSCs improved salivary gland function. We first examined whether the injected BMMSCs could migrate to the salivary glands. BMMSCs isolated from C57BL/6-gfp transgene mice were injected into NOD/Ltj mice and examined recipients’ salivary glands. One week after MSCs infusion, we observed green fluorescent protein (GFP) positive cells in the submandibular glands of the MSC-treated NOD/Ltj mice (Figure
3A, top right panel), and inflammation in affected salivary glands was also suppressed (not shown). We did not observe GFP positive cells in submandibular glands of normal control mice injected with GFP-BMMSCs (Figure 3A), indicating that the presence of GFP-BMMSCs in the submandibular glands was not due to a simple passive distribution, and the different microenvironment in NOD/Ltj mice versus normal ICR mice actively attracted BMMSCs to salivary glands.

Stromal cell-derived factor-1 (SDF-1) / C-X-C chemokine receptor 4 (CXCR4) axis has been recognized as an inflammatory chemokine that regulates MSCs trafficking 31. We measured the levels of SDF-1, and found that salivary gland had the highest concentration of SDF-1 in NOD/Ltj (Figure S2A), and NOD/Ltj mice had significantly higher levels of SDF-1 in serum ($P = 0.001$), salivary gland ($P = 1.287 \times 10^{-7}$), spleen homogenates ($P = 0.0002$) and lymph node ($P = 0.0004$) than ICR mice (n=6) (Figure 3B), suggesting the differential SDF-1 production may play a role in determining if BMMSCs trafficked into inflammation site. The reduction of CXCR4 (receptor for SDF-1) transcription in BMMSCs of NOD/Ltj mice was further confirmed by real-time PCR, and it was almost 5-fold lower than in BMMSCs from ICR, BALB/c or C57BL/6-gfp mice (Figure 3C), and CXCR4 in BMMSCs of SS patients was also about 8-fold lower than healthy human ($P = 0.009$, n=7; Figure 3D), suggesting that lower CXCR4 expression by BMMSCs from disease animals and patients, consequent failure to migrate into inflammation site, may play a role in their loss of immunoregulatory functions and consequent SS disease development.

To track migration of MSCs, we infused BMMSCs from C57BL/6-gfp mice to
NOD/Ltj, and found the distributions of GFP MSCs corresponded with their SDF-1 expression 1 week after infusion (Figures 3E and Figures S2C). Similar trends were also observed 1 day after infusion (Figure S2B). When treated BMMSCs from C57BL/6-gfp with CXCR4 neutralizing antibody before infusion, 0.38% ± 0.1% GFP positive cells were detected in salivary gland single cell suspensions in the non-blocked group at 1 week post treatment, but only 0.08% ± 0.05% GFP positive cells were detected in CXCR4-blocked group ($P = 4.5 \times 10^{-9}$, n=12; Figure 3E). Furthermore, CXCR4 blocking completely abolished effectiveness of BALB/c BMMSCs to restore secretory function of salivary glands in NOD/Ltj mice (Figure 3F). These data indicated that the SDF-1/CXCR4 axis is critical in directing BMMSCs to migrate towards inflammatory sites to control autoimmunity.

**MSC treatment favored Treg and Th2 while suppressing Th17 and Tfh responses**

We next investigated how MSCs directed CD4 T cell responses. We observed the frequency of Treg cells in the spleen of NOD/Ltj mice were far less than that of ICR control mice ($P = 2.597 \times 10^{-7}$, n=6; Figure 4A, B), and allogeneic BMMSCs from BALB/c mice significantly restored the number of Treg cells in NOD/Ltj mice ($P = 4.790 \times 10^{-9}$, n=6) in the spleen as early as 1 week. Similar results were also observed in submandibular lymph nodes (Figure S3A, B). In addition, when we performed experiments on splenectomized NOD/Ltj mice, we observed that allogeneic MSCs were similarly effective in inhibiting disease development (Figure S3C), and there
were similar higher levels of Treg cells in submandibular lymph nodes (Figure S3A, B), indicating that Tregs in spleen are not required for immune regulation and the therapeutic effects of MSCs likely occurred locally. When BMMSCs were treated with anti-CXCR4 Abs, the effect of Treg cell restoration was abrogated ($P = 2.397 \times 10^{-6}$, n=6). TGF-β and IL-10 are important for Treg cell proliferation and function.\textsuperscript{32-34} We found that the levels of TGF-β increased both in blood ($P = 0.035$, n=6) and salivary gland homogenates ($P = 0.034$, n=6; Figure 4C) in NOD/Ltj mice 1 week after BMMSC infusion, and blocking CXCR4 resulted in some reduction. Interestingly, IL-10 only increased in salivary gland homogenates ($P = 0.001$, n=6; Figure 4D), and CXCR4 blocking completely abolished these effects, suggesting that IL-10 may play a more direct and specific role for MSCs to control inflammatory responses in salivary gland than TGF-β (Figure 4C, D).

A predominance of Th1 rather than Th2 cell responses in patients with primary SS has been documented.\textsuperscript{35,36} We showed here that, although allogeneic BMMSC treatment did not suppress Th1 cells (IFN-γ) ($P = 0.262$, n=6; Figure 4E, F), it increased the percentage of Th2 cells (IL-4, IL-13) (all $P < 0.01$, n=6; Figure 4E, G and Figure S4A, B), and CXCR4-blocked BMMSCs failed to do so. CD4\textsuperscript{+}Th17 cells are involved during autoimmune diseases pathogenesis, and IL-6 participates in driving Th17 differentiation with or without TGF-β.\textsuperscript{25,37} We found that allogeneic BMMSCs significantly reduced the frequency of Th17 cells ($P = 1.386 \times 10^{-7}$, n=6), and blocking CXCR4 abolished this suppression in NOD/Ltj mice (Figure 4K, L). In addition, IL-6 was reduced in normal BMMSC-treated but not CXCR4-blocked
BMMSC-treated NOD/Ltj mice compared to untreated NON/Ltj mice, which correlated with the decrease in IL-17 levels in the same mice (Figure 4N, O), demonstrating that BMMSCs suppressed Th17 responses. T follicular helper T (Tfh) cells are a distinct subset of CD4⁺ helper T cells that regulate the development of antigen-specific B cell immunity. Upon exposure to a foreign antigen, Tfh cells help B cells generate antibody-producing plasma cells and long-lived memory B cells.³⁸ We next examined if MSCs regulated Tfh cells development. One week after allogeneic BMMSC treatment, the proportion of Tfh cells (CD4⁺CXCR5⁺, Figure 4K) decreased significantly (P =0.001, n=6; Figure 4M), and CXCR4 blocking reduced this inhibitory effect (P =0.053, n=6; Figure 4M). There was no difference in plasma cell concentration between groups (Figure 4P, Q), but SS-related autoantibodies (anti-nucleic antibody, anti-α-fodrin and anti SSA/Ro, Figure 4R-T) were significantly decreased in the serum of NOD/Ltj mice treated with normal BMMSCs while CXCR4 blocking partially abated autoantibodies suppression effects (Figure 4R-T). Together, these data collectively demonstrated that BMMSCs suppressed inflammatory responses by favoring Treg and Th2 differentiation while inhibiting Th17 and Tfh responses, further supporting that SDF-1/CXCR4 axis is critical for MSCs to exert immune regulatory activities.
Allogeneic MSC treatment suppresses diseases in patients with primary SS

Given the therapeutic effects of allogeneic MSCs on experimental SS in NOD/Ltj mice, we next performed a clinical evaluation of allogeneic MSC treatment on patients with primary SS. Twenty-four patients with primary SS (23 females and 1 male; age 45±12 years, range 27 - 68 years), were admitted to the hospital (Drum Tower Hospital of Nanjing University Medical School) and underwent umbilical cord mesenchymal stem cell treatment (MSCT) (Clinical Trial ID#: NCT00953485). Eleven patients reported symptoms of xerostomia and/or xerophthalmia and 13 presented with severe systemic comorbidities. Mean disease duration was 76.7 ± 82.5 months (range 3 - 384 months). All patients completed a 12-month follow-up, and clinical and laboratory data were collected before MSCT and at 2 weeks, 1, 3, 6, and 12 months post MSCT. The demographic and clinical manifestations were shown in Table S1.

All patients tolerated allogeneic MSCT well, and no adverse events occurred during or after MSCs infusion. All patients showed improvements in symptoms after MSCT, although with a different response time (from 2 weeks to 6 months). Mean SSDAI scores of all the 24 patients decreased from 5.63 ± 1.44 (baseline) to 4.58 ± 1.67 at 2 weeks, 4.33 ± 1.79 at 1 month, 4.08 ± 1.44 at 3 months, 3.46±1.18 at 6 months, and 3.08±1.21 at 12 months (all \( P < 0.05 \), Figure 5A). SSDAI scores of ~45.8% patients decreased >30% at 3 month from baseline, ~70.8% patients decreased >30% at 6 month from baseline, and at 12 month, approximately 83.3% of patients have their
SSDAI score decreased >30% from baseline. Global assessment by visual analog scale (VAS) also improved (Figure 5B) at 2 weeks \((P = 8.98 \times 10^{-6}, n=24)\) and showed further amelioration at 1, 3, 6, and 12 months after MSCT. At 3 month, 37.5% of patients have their VAS score decreased >30% from baseline, at 6 and 12 month, about ~58.3% and 75% of patients have their VAS score decreased >30% from baseline respectively.

Unstimulated salivary flow rate (Figure 5C) of all 11 patients with symptoms of xerostomia increased significantly 2 weeks after MSCT (versus baseline, \(P =0.0005, n=11\)) and showed a 2-fold increase at 1 month \((P =0.0098, n=11)\). This index continued to increase on subsequent follow-up visits. Stimulated salivary flow rate of these 11 patients also significantly increased at follow-up visits \((P =0.008, \text{at 2 weeks, } P = 0.043 \text{ at 1 month, } P = 0.016 \text{ at 3 months, } P = 0.017 \text{ at 6 months and } P = 0.016 \text{ at 12 months versus baseline, } n=11; \text{ Figure 5D})\). The determination of modified treatment emergent symptom scale (TESS) score (Figure 5E) decreased 2 weeks after MSCT \((P =0.05, n=11)\) and was maintained at this low level on subsequent visits.

For those with organ involvement at baseline, platelet counts increased significantly 2 weeks after MSCT for all 4 patients \([(85.75\pm37.92)\times10^3/\mu\text{l versus (37.50\pm22.19)\times10^3/\mu\text{l at baseline, } P =0.0001]}\), and continued to increase on subsequent visits \([(106.5\pm36.7)\times10^3/\mu\text{l at 1 month, (84.8\pm8.4)\times10^3/\mu\text{l at 3 months, (74.3\pm17.2)\times10^3/\mu\text{l at 6 months, (106.0\pm34.6)\times10^3/\mu\text{l at 12 months}]}\). Refractory hemolytic anemia also improved in all 3 patients after MSCT, hemoglobin level increased from 5.77\pm1.26 \text{ g/dl at baseline, to 7.13\pm0.61 \text{ g/dl at 2 weeks (} P =0.166),}
7.93±1.27 g/dl at 1 month (P =0.103), (8.23±1.3.82) g/dl at 3 months (P =0.079), (8.53±0.55) g/dl at 6 months (P =0.025), (8.87±1.33) g/dl at 12 months (P =0.043).

SS-related autoimmune hepatitis improved in all 7 patients, as shown by an improved liver function index. The level of alanine aminotransferase was 105.3±59.9 U/L before MSCT, 70.7±31.9 U/L at 2 weeks, 60.7±38.7 U/L at 1 month, significantly decreased at 3 months (49.9±20.0 U/L, P =0.039), 6 months (42.3±24.8 U/L, P =0.025) and 12 months (50.43±19.78 U/L, P =0.04). The concentration of aspartate aminotransferase was 130.8±45.9 U/L at baseline, 99.3±34.9 U/L at 2 weeks, 79.0±33.1 U/L at 1 month, significantly decreased at 3 months (61.5±20.0 U/L, P =0.007), 6 month (45.2±12.2 U/L, P =0.01) and 12 months (38.5±11.17 U/L, P =0.01).

For one patient with refractory enteritis, diarrhea improved after MSCT, in parallel with increased body weight. Nervous system involvement in 3 patients (myelitis, leukodystrophy and peripheral nervous system involvement, respectively) showed no satisfactory improvements after MSCT. Medications used for each patient were tapered according to disease amelioration. Two patients discontinued immunosuppressive drugs and one patient had prednisone withdrawn 6 months after MSCT, with sustained disease remission. The treatment protocol for each patient pre and post MSCT was shown in Table S2.

We investigated whether MSCT regulated the immune response in SS patients. We observed MSCT completely abolished production of anti-SSA/Ro (Figure 5F) in serum, decreased from 84.76 ± 62.19 unit/ml at baseline to 0.51 ± 0.22 unit/ml 1 month after treatment (n=7); and also resulted in more than 50% of inhibition of
anti-SSB/La (Figure 5G) in serum, decreased from 146.62 ± 83.08 unit/ml to 52.61 ± 38.67 unit/ml (n=6), further suggesting the important role of MSCs in suppressing Tfh differentiation and function. Together, these data demonstrated that UCMSCs treatment substantially increased salivary flow rate, ameliorated disease symptoms, and inhibited the inflammatory responses, and allogeneic MSC treatment is a novel, effective, and safe therapy for the patients with SS.

Discussion

In the present study, we revealed for the first time that immunoregulatory functions and biological properties of MSCs in SS patients are significantly impaired. We found that treatment of allogeneic MSCs prevented and suppressed experimental SS-like diseases in NOD/Ltj mice. More importantly, we showed a novel therapeutic approach to alleviate diseases in patients with primary SS by infusing allogeneic UCMSCs. We demonstrated that the therapeutic effects of MSC treatment were attributed to their immunoregulatory activities in driving CD4 T cells favoring Treg and Th2 development while inhibiting Th17 and Tfh inflammatory responses. Notably, we also discovered a critical role of SDF-1/CXCR4 axis in directing, MSCs trafficking towards inflammation sites, to exert suppressive activities and improve salivary gland function.

MSCs have been successfully utilized to treat a variety of human diseases for their wide-ranging differentiation potential, possibility of engraftment and immunoregulatory effects. MSCs are reported to modulate immune responses through
multiple mechanisms, including prostaglandin E2 (PGE2), nitric oxide (NO), indoleamine 2,3-dioxygenase (IDO), and toll-like receptor (TLR) signaling pathways. Exposure to IFN-γ did not ablate MSC inhibited T cell proliferation, but rather antagonized TGF-β suppressed allo-responsiveness. MSCs induce Treg cells, thereby interfering with the immune response to alloantigens. For local immunity, inflammation following tissue damage can recruit MSCs into the inflamed sites where they participate in tissue regeneration and modulation of immune response. SDF-1/CXCR4 interaction played a critical role in directing hematopoietic cells and MSCs trafficking in vivo. In our study, we observed that GFP MSCs distributed most in the kidney and lung, followed by spleen and liver at day 1 post-infusion both in NOD/Ltj and ICR mice, suggesting that GFP MSCs at 1 day after infusion was not only affected by SDF-1 concentration but also by blood supply and histological situation. Then distributions in kidney, spleen and lung both in NOD/Ltj and ICR at 1 week post infusion were decreased, but GFP cells in salivary gland of NOD/Ltj remained at high level, corresponded well with the SDF-1 expression level, further demonstrating the critical role of SDF-1 in directing MSCs migrating towards inflammatory salivary glands to exert their immunoregulatory functions. This notion was supported by our data that blockade of CXCR4 in BMMSCs with specific antibody abrogated the immunoregulatory activity and consequently the therapeutic effects of normal BMMSCs. Moreover, we found the similar higher level of Treg cells in submandibular lymph nodes after MSC treatment with or without spleen, and allogeneic MSCs were similarly effective in inhibiting disease development in
splenectomized NOD/Ltj mice, indicating that Treg cells in spleen were not required for immune regulation and the therapeutic effects MSCs likely occurred locally.

Infusion of MSCs from bone marrow is considered safe and has been widely tested in clinical trials of cardiovascular, neurological, and immunological disease with encouraging results.\textsuperscript{20,42,43} Compared to BMMSCs, UCMSCs have significant advantages as a cellular source for MSC-mediated therapy. First, collection from the umbilical cord is easy and non-invasive for the donor;\textsuperscript{44} secondly, umbilical cords can be stored in advance, while bone marrows specimens have to be collected from the donor immediately before infusion; thirdly, MSCs from the umbilical cord are more primary than MSCs isolated from some other tissue sources.\textsuperscript{45} Finally, UCMSCs have greater proliferative potential and possess therapeutic effects in experimental and human autoimmune diseases.\textsuperscript{21,46} Clinically, treatment for hypofunction of salivary gland in primary SS remained a challenge, long-term use of sialogogue can only alleviate xerostomia and usually be with adverse effects. Here, we showed that treatment of UCMSCs in 24 patients with primary SS resulted in considerable improvements in disease activity and organ function, demonstrating that UCMSCs as a reliable cellular source of MSCs, and their infusion as a novel therapeutic approach for SS.

In summary, our study showed for the first time that allogeneic MSC infusion is an effective treatment for SS, and has important implication for further exploration of MSCs as a novel therapy for patients with SS and other autoimmune diseases. Our data demonstrate that SDF-1-mediated MSCs migration plays a key role in their
immunoregulatory functions and controlling SS and other autoimmune diseases pathogenesis.
Contributors

S.W. was responsible for the concept and design of the study and obtained funding. J.X. and D.W. were responsible for acquisition of data. J.X. performed the statistical analysis. S.W., L.S., Y.D., J.B. and W.C. supervised the study. Z.F., H.Z. and C.Z. provided administrative, technical, and material support. S.W., L.S., J.X. and Y.D. drafted the manuscript. All authors participated in the analysis and interpretation of data critical revision of the report for intellectual content and provided final approval of the submitted version.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. Impairment of immunoregulatory activity of BMMSCs during SS pathogenesis. (A, B) BMMSCs from NOD/Ltj mice failed to effectively suppress the proliferation of stimulated T cells. Stimulated normal T cells co-cultured with NOD BMMSCs showed higher proliferative response compared to those co-cultured with control ICR BMMSCs \( (P = 0.0002, n=12) \). (C, D, E) CD4^+Foxp3^+Treg cells from splenocytes co-cultured with ICR BMMSCs or NOD BMMSCS. CD4^+ cells in splenocytes co-cultured with NOD/Ltj BMMSCs was significantly higher than that with ICR BMMSCs \( (* P = 0.0005, n=12) \), ICR BMMSCs had better regulatory potential for Treg cells compared to BMMSCs from NOD mice \( (* P = 0.0001, n=12) \). (F, G) Impairment of immunoregulatory capacity was observed in BMMSCs of SS patients. Normal PBMCs cultured with BMMSCs obtained from SS patients showed higher proliferative response compared to those co-cultured with normal BMMSCs \( (P = 0.01, n=5) \).

Figure 2. MSC treatment reduced inflamed tissue damage and improved salivary gland function in NOD/Ltj mice. (A, B, C) Histology of submandibular glands of NOD/Ltj mice in untreated, prevention and treatment groups. Pre-treated: 6 week for prevention and 16 week for treatment groups; 2 wk post: 2 week after with or without MSCs infusion (8 wk for prevention and 18 wk for treatment groups. Yellow box area is magnified in black box. In the prevention group (B), the infiltrating area in submandibular glands was significantly smaller than control \( (P =0.017, n=6) \). In the
treatment group (C), the infiltrating area was significantly reduced 2 weeks post-MSC infusion ($P =0.009$, n=6). (D, E) The saliva flow rate of NOD/Ltj mice before and after MSCs infusion. The saliva flow rate began to decrease at about 6 weeks of age, and declined rapidly at 7 and 8 weeks of age (* 6, 7, 8 weeks versus 4 weeks in untreated group, all $P <0.05$, n=6). In the prevention group (D), the saliva flow rate returned to baseline (4 weeks level) 2 weeks post-BMMSC infusion ($^\# P =0.977$, n=6). (E) The saliva flow rate in the treatment group increased 2 weeks post-BMMSC infusion ($P =0.045$, n=6), while the untreated group remained at a lower level.

**Figure 3. A critical role of SDF-1/CXCR4 for MSCs trafficking and anti-inflammatory functions.** (A) GFP positive cells were detected in the submandibular glands of NOD/Ltj mice but not in control mice 1 week after allogeneic GFP-labeled BMMSC infusion. (B) ELISA (n=6) showed that SDF-1 was significantly higher in serum ($^\delta P =0.001$), salivary gland ($^\delta P =1.287\times10^{-7}$), spleen ($^\delta P =0.0002$) and lymph nodes homogenates ($^\delta P =0.0004$) of NOD/Ltj mice than in control mice. In NOD/Ltj, the concentration of SDF-1 in salivary gland was the highest (* all $P <0.05$), and in ICR mice bone marrow contained the highest SDF-1 ($^\#$ all $P <0.05$). (C) Real time PCR for Cxcr4 expression in mice BMMSCs. The level of BMMSCs Cxcr4 gene from NOD/Ltj mice of 0.082 ± 0.043, was about 5 fold lower than that from ICR, BALB/c or C57BL/6-gfp mice (* all $P <0.05$, n=9). (D) Real time PCR for CXCR4 expression in human BMMSCs. The level of BMMSCs CXCR4 gene from SS patients was about 8 fold lower than that from healthy people ($P =0.009$,
n=7). (E) In NOD/Ltj mice, higher numbers of GFP+ BMMSCs were detected in salivary glands post C57BL/6-gfp MSCs infusion (MSCT NOD) compared to the CXCR4-blocked C57BL/6-gfp BMMSCs group (C-b MSCT NOD) 1 week post-transplantation ($P = 4.5 \times 10^{-9}$, n=12). A similar trend was observed for GFP+ BMMSCs in spleen, bone marrow, lymph node in both NOD/Ltj and ICR mice (* all $P < 0.05$, n=12). (F) Salivary flow rate (n=6) of CXCR4-blocked BALB/c BMMSCs treated NOD/Ltj mice was significantly lower than mice in normal BALB/c BMMSCs infusion group (* $P = 0.044$ at 17 weeks and $P = 0.036$ at 18 weeks) and were similar to untreated control group (# $P = 0.475$ at 17 weeks and $P = 0.522$ at 18 weeks).

**Figure 4. MSC treatment mediated immune regulation.** (A-D) Treg and related cytokines. (A) CD25 and FoxP3 in CD4 T cells of splenocytes in ICR control mice (ICR), NOD/Ltj untreated mice (Untreated), BALB/c BMMSCs infused NOD/Ltj mice (MSC Treated) and CXCR4-blocked BALB/c BMMSCs infused NOD/Ltj mice (C-b MSC Treated). (B) Treg in spleen of NOD/Ltj untreated mice were far less than control ICR mice ($P = 2.597 \times 10^{-7}$, n=6), and allogeneic BMMSC partly restored Treg cells ($P = 4.790 \times 10^{-9}$, n=6), but CXCR4 blocking reduced MSCs mediated Treg generation compared to normal BMMSCs ($P = 2.397 \times 10^{-6}$, n=6). (E-J) Th1, Th2 and related cytokines, Intracellular staining for IFN-$\gamma$ and IL-4 in CD4+ splenocytes. (E) Higher numbers of Th1 cells were observed in NOD/Ltj untreated mice than control ICR mice (F) ($P = 7.518 \times 10^{-5}$, n=6), and there was no change of Th1 response 1 week after allogeneic BMMSC or CXCR4-block BMMSC infusion. Th2 responses in the
spleen of NOD/Ltj untreated mice were less than that of control ICR mice (G) \((P = 6.917 \times 10^{-6}, n=6)\). Allogeneic BMMSC could partly restore Th2 \((P = 0.0008, n=6)\) and blocking of CXCR4 abolished this effect \((P = 0.323, n=6)\). (K-O) Th17, Tfh, and related cytokines. Flow cytometry for CXCR5 (Tfh) and IL-17 (Th17) expression in CD4 splenocytes. (K) Th17 were significantly higher in NOD/Ltj untreated mice than in control ICR mice \((P = 0.0004, n=6)\). Allogeneic BMMSC mitigated the percentage of Th17 \((P = 1.386 \times 10^{-7}, n=6)\), and CXCR4 blocking resulted in less suppression \((P = 0.025, n=6)\). (M) MSC treatment suppressed Tfh response. Allogeneic BMMSC regulated T cell cytokines IFN-\(\gamma\) (H) and IL-17 (O), regulatory cytokines IL-10 (D), IL-13 (I) and IL-4 (J), and other cytokines TGF-\(\beta\) (C) IL-6 (N) production in serum and/or salivary gland homogenates. Blocking of CXCR4 of BMMSCs resulted impairment of immunoregulatory activities. (P-T) Plasma cells and autoantibodies, CD19 and CD138 gated splenocytes in the four groups. (P) There was no significant difference of plasma cells in these four groups (Q). Both allogeneic BMMSC and CXCR4 blocked BMMSC transplantation decreased the SS related autoantibodies - anti nucleic antibody (R), anti \(\alpha\)-fodrin (S) and anti SSA/Ro (T) in serum.

**Figure 5. Allogeneic UCMSC treatment improves salivary gland function and suppresses disease activity and autoimmunity in Sjögren’s syndrome patients.** (A) SSDAI scores for all 24 patients decreased 2 weeks, 1, 3, 6, and 12 months after UCMSCT \((all \ P < 0.05)\). (B) The VAS of all 24 patients decreased significantly after
UCMSCT (all \( P < 0.05 \)). Unstimulated (C) and stimulated (D) salivary flow rate of the 11 patients with xerostomia increased significantly at 2 weeks after UCMSCT (all \( P < 0.05 \)) and maintained this level at 1, 3, 6, and 12 months follow up. (E) The TESS score of the 11 patients with xerostomia decreased 2 weeks after UCMSCT (\( P = 0.05 \)) and maintained at this low level on subsequent visits. (F,G) Allogeneic UCMSCT regulated SS related autoantibodies in serum. (F) Anti-SSA/Ro, decreased from 84.76 ± 62.19 unit/ml at baseline to 0.51 ± 0.22 unit/ml 1 month after treatment (n=7), and (G) anti-SSB/La decreased from 146.62 ± 83.08 unit/ml to 52.61 ± 38.67 unit/ml (n=6) 1 month after treatment.
Figure 1
Figure 2

A

Prevention group

Treatment group

Pre-treatment

6 wk

16 wk

8 wk

18 wk

Unreacted

Treated

2 wk post

B

Prevention group

Treatment group

Infiltrating area (Kruskal-Wallis test)

Pre-treatment

2 wk post

C

Infiltrating area (Kruskal-Wallis test)

Pre-treatment

2 wk post

D

Prevention group

Salivary flow rate (mg/10 ml)

4 wk

6 wk

7 wk

8 wk

E

Treatment group

Salivary flow rate (mg/10 ml)

4 wk

18 wk

17 wk

18 wk

Figure 3

A

NOD SG

ICR SG

GFP

GFP

B

ICR

Serum

salivary gland

serum

lymph node

bone marrow

ICR

NOD

C

D

E

F

GFP levels (ng/ml)

Salivary gland

Spleen

Bone marrow

Lymph node

Salivary flow rate (mg/10 ml)

15 wk

17 wk

18 wk
Figure 4
Figure 5
Allogeneic mesenchymal stem cell treatment alleviates experimental and clinical Sjögren’s syndrome

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