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Original Article

Dental pulp-derived stem cell-conditioned media attenuates secondary Sjögren's syndrome via suppression of inflammatory cytokines in the submandibular glands

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ABSTRACT

Introduction: Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease, which affects the exocrine glands. Its primary symptoms are decreased moisture in the mouth and eyes. Therapies are limited to treatment with steroids, which has unpleasant side effects, so new treatments would be beneficial. One possibility might be stem cells, such as bone marrow mesenchymal stem cells (BMMSCs) or dental pulp-derived stem cells (DPSCs); these have been reported to exert immunomodulatory effects on activated lymphoid cells. This study aimed to evaluate the effects of conditioned media from DPSCs (DPSC-CM) or BMMSCs (BMMSC-CM) on salivary functions in SS.

Methods: Cytokine array analysis was performed to assess the types of cytokines present in the media. DPSC-CM or BMMSC-CM was administered in an SS mouse model. Histological analysis of the salivary glands was performed, and gene expression levels of inflammatory and anti-inflammatory cytokines in the submandibular glands (SMGs) were evaluated.

Results: DPSC-CM contained more anti-inflammatory factors than BMMSC-CM. The mice that were given DPSC-CM had a lower number of inflammation sites in the SMGs than those in the other experimental groups, and their salivary flow rate increased. The expression levels of *interleukin (IL)-10* and *transforming growth factor-β1* increased in the DPSC-CM group, while those of *Il-4*, *Il-6*, and *Il-17a* decreased. The mice that received DPSC-CM showed a significantly increased percentage of regulatory T cells and a significantly decreased percentage of type T helper 17 cells compared to other groups.

Conclusions: These results indicate that DPSC-CM could be an effective therapy for SS-induced hyposalivation, since it decreases the number of inflammatory cytokines and regulates the local inflammatory microenvironment in the SMGs.

1. Introduction

Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease that interferes with the functions of the exocrine glands and is marked by decreased moisture secreted from the mucous glands of the mouth and eyes [1]. This disease is more common in middle-aged women; it can occur alone (primary SS) or in association with other rheumatic diseases (secondary SS), such as rheumatoid arthritis, systemic sclerosis, polymyositis, mixed cryoglobulinemia, and systemic lupus erythematosus (SLE) [2].

SS is most often associated with SLE and occurs in 9%–33% of SLE patients [3–5]. The most prominent feature of SLE is the production of multiple circulating autoantibodies; some reactivities, such as the anti-double-stranded DNA (dsDNA), anti-Sjögren's syndrome A (SSA), and anti-Sjögren's syndrome B (SSB) antibody, are specific serologic markers of SLE [6]. The diagnostic boundaries between primary SS and SLE can be difficult to discern because they share clinical and laboratory features.

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Three drugs are currently licensed for the treatment of lupus: corticosteroids, hydroxychloroquine, and belimumab [7]. Immunosuppressants, such as azathioprine, methotrexate, and mycophenolate, are also used; despite these treatments, however, lupus patients face serious morbidity. Steroids are not likely to cause serious side effects if they are taken for a short time or at a low dose [8], but there are unpleasant minor unpleasant side effects, such as an increased appetite, mood changes, and insomnia. Therefore, new treatments are needed for the management of the secondary SS consequences of active lupus.

Extensive clinical trials have recently been conducted using mesenchymal stem cell (MSC)-based therapies for treating a range of diseases [9–11]. Studies have shown that transplanted cells can play multiple and important roles; they can not only migrate in their host tissues and participate directly in the regeneration of tissue but also display paracrine effects [12–15]. MSCs produce cytokines that can decrease inflammation, enhance progenitor cell proliferation, improve tissue repair, and decrease infection.

Studies have shown that dental pulp-derived stem cell-conditioned media (DPSC-CM) has immunoregulatory properties that contribute to tissue repair and anti-inflammatory effects comparable to bone marrow-derived mesenchymal stem cell-conditioned media (BMMSC-CM) [16]. In this study, we will evaluate the therapeutic effects of DPSC-CM and BMMSC-CM in mouse models of secondary SS.

2. Materials and methods

2.1. Ethics statement

The study design and methods were approved by and carried out according to the Institutional Review Board of the Center for Clinical and Translational Research of Kyushu University Hospital (IRB serial numbers 25–287 and 26–86) and the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients or their relatives prior to inclusion in the study.

All animal protocols were approved by the Animal Experiments Care and Use Committee of Kyushu University (approval nos. A19-060-0 and 29–104). Maximum efforts were made to minimize animal suffering; all measurements under catheter insertion were performed with the animals under deep anesthesia.

2.2. Cell preparation

Human DPSCs and BMMSCs were purchased from Lonza, Inc. (Walkersville, MD, USA). DPSCs were cultured in DPSC basal medium (Lonza, Inc.) containing DPSC SingleQuots (Lonza, Inc.) at 37 °C in 5% CO_2 and 95% air. The BMMSCs were cultured in MSC basal medium (Lonza, Inc.), containing MSC-GM SingleQuots (Lonza, Inc.), at 37 °C in 5% CO_2 and 95% air. After primary culture, the cells were subcultured at a density of approximately 1×10^4 cells/cm^2. Cells from the third to sixth passages were used for the experiments.

2.3. Preparation of CM

After achieving 80% confluence, the DPSCs or BMMSCs were replenished with serum-free Dulbecco's Modified Eagle's Medium (DMEM (−); Gibco, Rockville, MD, USA) containing an antibiotic–antimycotic solution. The cell-cultured CM was collected after 48 h of incubation and centrifuged at 440 × *g* for 5 min at 4 °C. The supernatant was collected, centrifuged at 17,400 × *g* for 3 min at 4 °C, and filtered using 0.22 μm pore filters (Millex-GP; Merck Millipore Ltd., Billerica, MA, USA). The DPSC-CM and BMMSC-CM were stored at −80 °C before use in the experiments.

2.4. Cytokine antibody array

Cytokine array analysis was performed via laser scanning using 174 human-cytokine array plates to assess the cytokines present in the DPSC-CM and BMMSC-CM (Quantibody® Human Cytokine Array 6000; RayBiotech, Inc., Norcross, GA, USA). Each scan was performed in duplicate, and data were calculated as the ratio of the cytokine levels in DPSC-CM to that in BMMSC-CM.

2.5. Mice model and injection of DPSC-CM or BMMSC-CM

We used 10-week-old MRL/MpJ-faslpr/faslpr (MRL/*lpr*) female mice (Charles River Laboratories Japan [Yokohama, Japan]) as the model of secondary SS. The MRL/*lpr* mouse is an autoimmune strain that develops lacrimal and salivary gland inflammation (dacryoadenitis and sialadenitis) and is a model for human SS [17]. The mice were divided into four treatment groups, each receiving intravenous injections twice a week ($n = 6$ per group): (1) non-treatment group, (2) DMEM (−) group (500 μL injections), (3) BMMSC-CM group (500 μL injections), and (4) DPSC-CM group (500 μL injections). The mice were euthanized 2 weeks after the intravenous injections.

2.6. Measurement of stimulated saliva flow

The MRL/*lpr* mice were anesthetized with chloral hydrate (0.4 g/kg body weight), and the stimulated saliva flow was measured as previously described [18,19]. At 3 min after intraperitoneal injection of pilocarpine (0.05 mg/100 g body weight), a micropipette was used to collect whole saliva from the oral cavity for 10 min, and the amount of saliva collected was then calculated.

2.7. Histological analysis

Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed as previously described [12]: dissected submandibular glands (SMGs) were fixed in 4% paraformaldehyde, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. The samples were cut to create 5-μm-thick histological sections, which were stained with H&E and analyzed under a light microscope.

To clearly assess the inflammation, focus scores were analyzed. The ratio of the foci area to the total area of SMGs was calculated and presented (six independent experiments for each group).

2.8. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed (Click-iT Plus TUNEL Assay with Alexa Fluor 647; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to detect apoptotic cells. Images of the sections were taken with a fluorescence microscope (BZ-X810; Keyence, Osaka, Japan; n = 10 per group). We calculated the percentage of TUNEL-positive cells per total number of cells in the SMGs of each group.

2.9. Enzyme-linked immunosorbent assay (ELISA) analysis

The concentrations of anti-dsDNA and anti-SSA in the mice were measured using mouse anti-dsDNA and mouse anti-Ro52/SSA ELISA kits (Signosis, Inc., Santa Clara, CA, USA). The serum samples were diluted at a ratio of 1:50.

2.10. Extraction of RNA and synthesis of complementary DNA (cDNA)

Total RNA isolated from the SMGs (n = 6 per group) was dissected with a QIAshredder and RNeasy Mini Extraction Kit (QIAGEN, Hilden, Germany) as previously described [20]. One microgram of total RNA was prepared and used for cDNA synthesis. The RNA was incubated for 1 h at 42 °C with 20 units of RNase inhibitor (Promega Japan, Tokyo, Japan), 0.5 μg of Oligo(dT)$_{12\text{-}18}$ primer (Thermo Fisher Scientific, Inc.), 0.5 mM deoxyribonucleotide triphosphate (AB0196; Thermo Fisher Scientific, Inc.), 10 mM dithiothreitol, and 100 units of RNA reverse transcriptase (Life Technologies Japan, Ltd., Tokyo, Japan).

2.11. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was used to determine the mRNA levels of the cytokines. The resulting cDNA was amplified using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, Inc.) and the AriaMX Real-Time PCR instrument (version 1.7; Agilent Technologies, Inc., Santa Clara, CA, USA). We analyzed the mRNA levels of *interleukin* (*Il*)*-2*, *interferon* (*Ifn*)*-γ*, *Il-10*, *Il-4*, *Il-6*, *Il-17a*, and *transforming growth factor* (*Tgf*)*-β1*.

Target mRNA levels were expressed relative to β-actin (housekeeping gene), using the $2^{-\Delta\Delta CT}$ method for the analyses (performed in triplicate). The following PCR primers were used for further specific analysis: *Il-2*, 5′-ACTGTTGTAAAACTAAAGGGCTCTG-3′ and 5′-GCAGGAGGTACATAGTTATTGAGGG-3′; *Ifn-γ*, 5′-CTTGGCTTTGCAGCTCTTCC-3′ and 5′-CACATCTATGCCACTTGAGTTAAAA-3′; *Il-4*, 5′-TCTTTCTCGAATGTACCAGGAGC-3′ and 5′-TGTGAGGACGTTTGGCACATC-3′; *Il-6*, 5′-AGTTCCTCTCTGCAAGAGACTTC-3′ and 5′-TTTCCACGATTTCCCAGAGAAC-3′; *Il-17a*, 5′-CAGGGAGAGCTTCATCTGTGTCTC-3′ and 5′-TGCGCCAAGGGAGTTAAAGAC-3′; *Il-10*, 5′-GGTAGAAGTGATGCCCCAGG-3′ and 5′-AATCGATGACAGCGCCTCAG-3′; *Tgf-β1*, 5′-CAGGGAGAGCTTCATCTGTGTCTC-3′ and 5′-TGCGCCAAGGGAGTTAAAGAC-3′; and *β-actin*, 5′-CACTCCTAAGAGGAGGATGGTCG-3′ and 5′-CAGACCTGGGCCATTCAGAAA-3′.

2.12. Immunohistochemical analysis

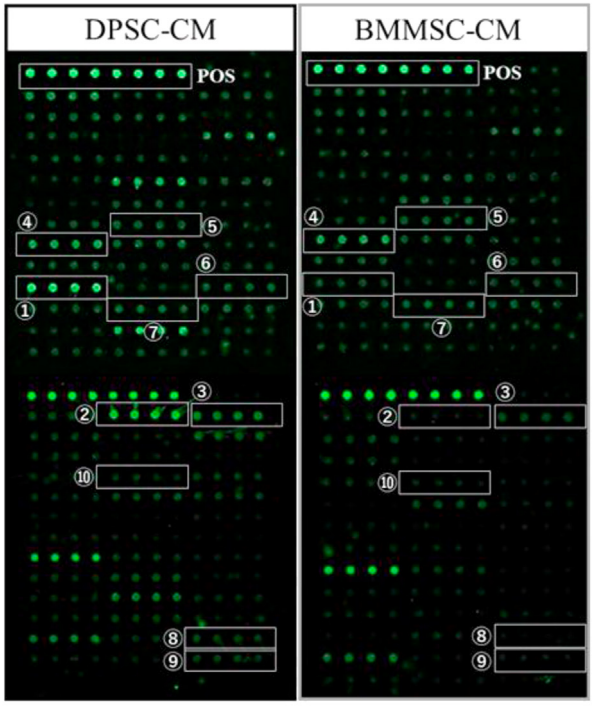
Immunohistochemical staining was performed for T-bet (1:500; sc-21763; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) to evaluate Th1 cells; GATA binding protein 3 (GATA3; 1:500; sc-268; Santa Cruz Biotechnology, Inc.) was used for the Th2 cells; forkhead box protein P3 (FOXP3; 1:200; NB100-39002; Novus Biologicals, Centennial, CO, USA) was used for regulatory T (Treg) cells; and RAR-related orphan receptor (ROR)γ (1:1000; ab207082; Abcam, Cambridge, UK) was used for Th17 cells.

The sections were rehydrated, subjected to antigen retrieval using Dako Target Retrieval Solution (pH 9.0; Dako North America, Inc., Carpinteria, CA, USA) for 10 min at 121 °C, blocked for endogenous peroxidase with 0.3% H_2O_2 in methanol, and incubated for 30 min. After washing with phosphate-buffered saline, the sections were blocked for nonspecific binding using Blocking One Histo (Nacalai Tesque, Inc., Kyoto, Japan) for 15 min at room temperature and then incubated with the primary antibody overnight at 4 °C. The sections were reacted using peroxidase stain 3,3-diaminobenzidine (DAB) kit (Nacalai Tesque, Inc.) for 1 h and developed with DAB solution. Hematoxylin counterstaining was performed following the DAB reaction.

2.13. Statistical analysis

All experiments were conducted in triplicate and repeated at least twice. Group means and standard deviations were calculated for each measured parameter. Statistical differences were evaluated using the Student's t-test, Mann–Whitney U test, and Tukey's honest significant difference test. A p-value < 0.05 was considered to be statistically significant, and a p-value of <0.01 was considered to be highly significant.

a



b

	Anti-inflammatory factors (Intensity)	
	DPSC-CM	BMMSC-CM
① TGF-β1	11623	2186
② IL-10	7989	234
③ IL-13	5098	80
④ IGF-1	3521	3324
⑤ TECK	1609	1513
⑥ MCP-1	965	483
⑦ IL-29	943	1019
⑧ Adiponectin	502	0
⑨ Siglec-9	396	0
⑩ GM-CSF	159	188

Fig. 1. DPSC-CM contains more anti-inflammatory factors than BMMSC-CM. (a) Images of the multiplexed sandwich ELISA-based quantitative array (POS, positive control). (b) The anti-inflammatory factors of DPSC-CM vs. MSC-CM. *Abbreviations*: TGF-β1: transforming growth factor-β1; IL-10: interleukin-10; IL-13: interleukin-13; IGF-1: insulin-like growth factor-1; TECK: thymus-expressed chemokine; MCP-1: monocyte chemoattractant protein-1; IL-29: interleukin-29; Siglec-9: sialic acid-binding immunoglobulin-type lectin-9; GM-CSF: granulocyte macrophage colony-stimulating factor.

3. Results

3.1. DPSC-CM contains more anti-inflammatory cytokines than BMMSC-CM

As shown in Fig. 1a and b, ten representative anti-inflammatory cytokines were selected. DPSC-CM contained more anti-inflammatory cytokines than BMMSC-CM (TGF-β1, ×5; IL-10, ×34; and IL-13, ×63) (Fig. 1b).

3.2. DPSC-CM prevents a decrease in saliva and inhibits inflammation in SMGs

Our protocols for the administration of DMEM (−), BMMSC-CM, and DPSC-CM are shown in Fig. 2a. As shown in Fig. 2b, DPSC-CM alleviated inflammation in the SMGs of the mice, and the focus scores in the DPSC-CM group were lower than those in the non-treatment, DMEM (−), and BMMSC-CM groups (Fig. 2c).

The stimulated saliva flow rate increased in the 12-week-old mice injected with DPSC-CM than that in the other groups (Fig. 2d). Interestingly, anti-dsDNA and anti-Ro52/SSA were highly detected in the serum collected from the non-treated, DMEM (−), or

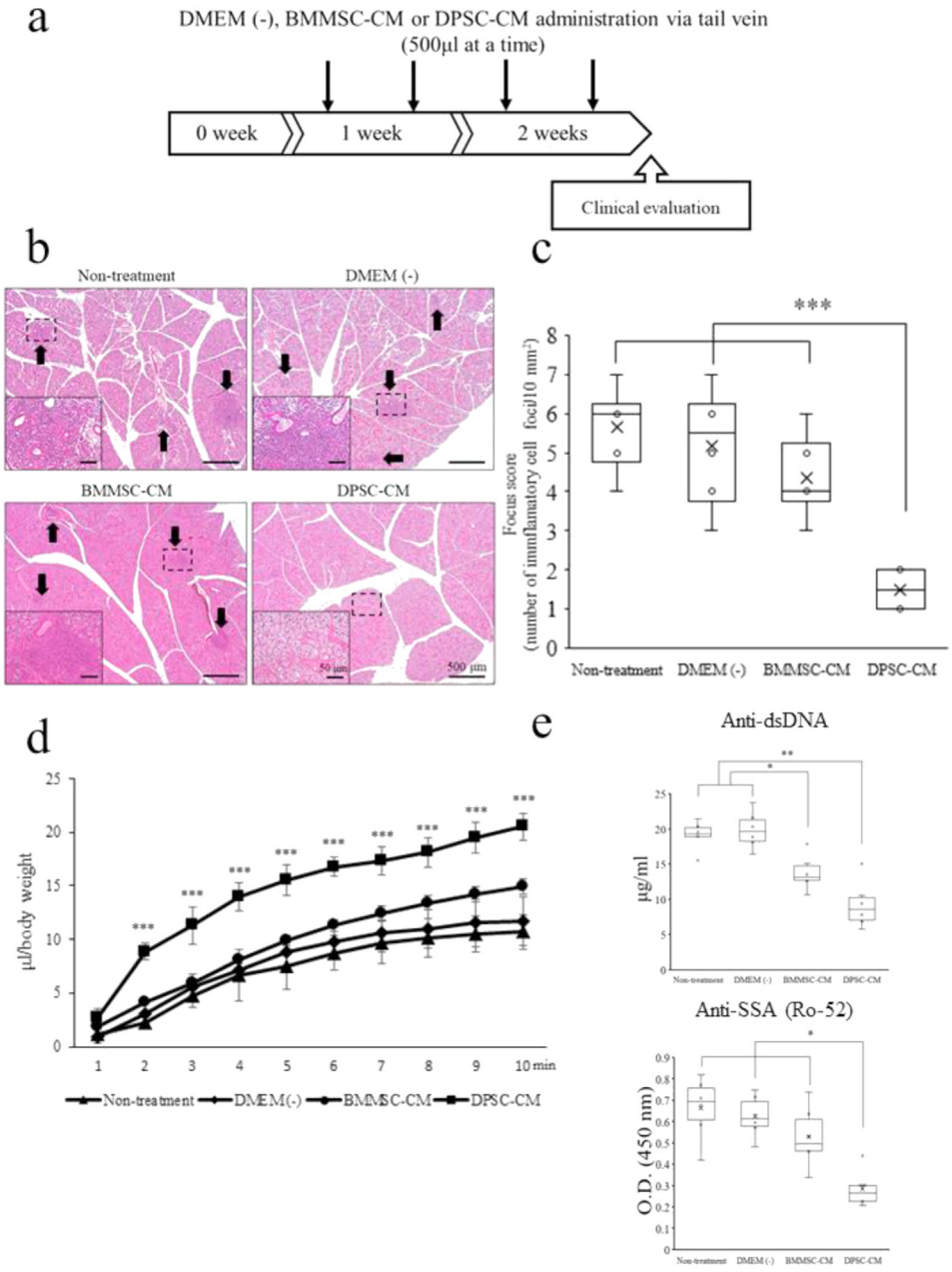


Fig. 2. Evaluation of inflammatory infiltration in the SMGs of MRL/*lpr* mice. (a) The study protocol. (b) Representative histological images of the SMGs for H&E staining in 12-week-old MRL/*lpr* mice. The bars of lower magnifications are 500 μm, and those of higher magnifications are 50 μm. (c) The degree of inflammatory infiltration in the SMGs. Data are representative of the mean ± standard deviation ($n = 6$. ***$p < 0.001$). (d) The salivary flow rate in each group at 12 weeks of age ($n = 6$ per group, ***$p < 0.001$). (e) Quantification of anti-dsDNA (top) and anti-SSA/Ro-52 (bottom) antibodies in the MRL/*lpr* mice at 12 weeks of age. Data represent the mean ± standard deviation ($n = 6$, **$p < 0.01$, *$p < 0.05$).

BMMSC-CM-administered mice than those in the DPSC-CM group (Fig. 2e).

3.3. Relative mRNA expression levels of inflammatory or anti-inflammatory cytokines in the SMGs

The SMGs of mice who received DPSC-CM had significantly decreased relative mRNA expression levels of inflammatory cytokines, such as *Il-2*, *Ifn-γ*, *Il-4*, *Il-6*, and *Il-17a* (Fig. 3a). On the other hand, levels of *Il-10* and *Tgf-β1*, both anti-inflammatory cytokines, were significantly increased in these mice (Fig. 3a).

3.4. DPSC-CM administration decreases the number of apoptotic cells in the SMGs

We then investigated apoptotic cells and found that they increased in the SMGs of the non-treatment and DMEM (−) groups than the BMMSC-CM and DPSC-CM groups (Fig. 3b). The number of apoptotic cells significantly decreased in the group receiving DPSC-CM relative to the group receiving BMMSC-CM (Fig. 3b).

3.5. DPSC-CM induces FOXP3-expressing cells and inhibits RORγ-expressing cells in the mouse spleen

We investigated the T helper subset in the mouse spleen tissue to confirm the mechanism by which DPSC-CM influences the whole

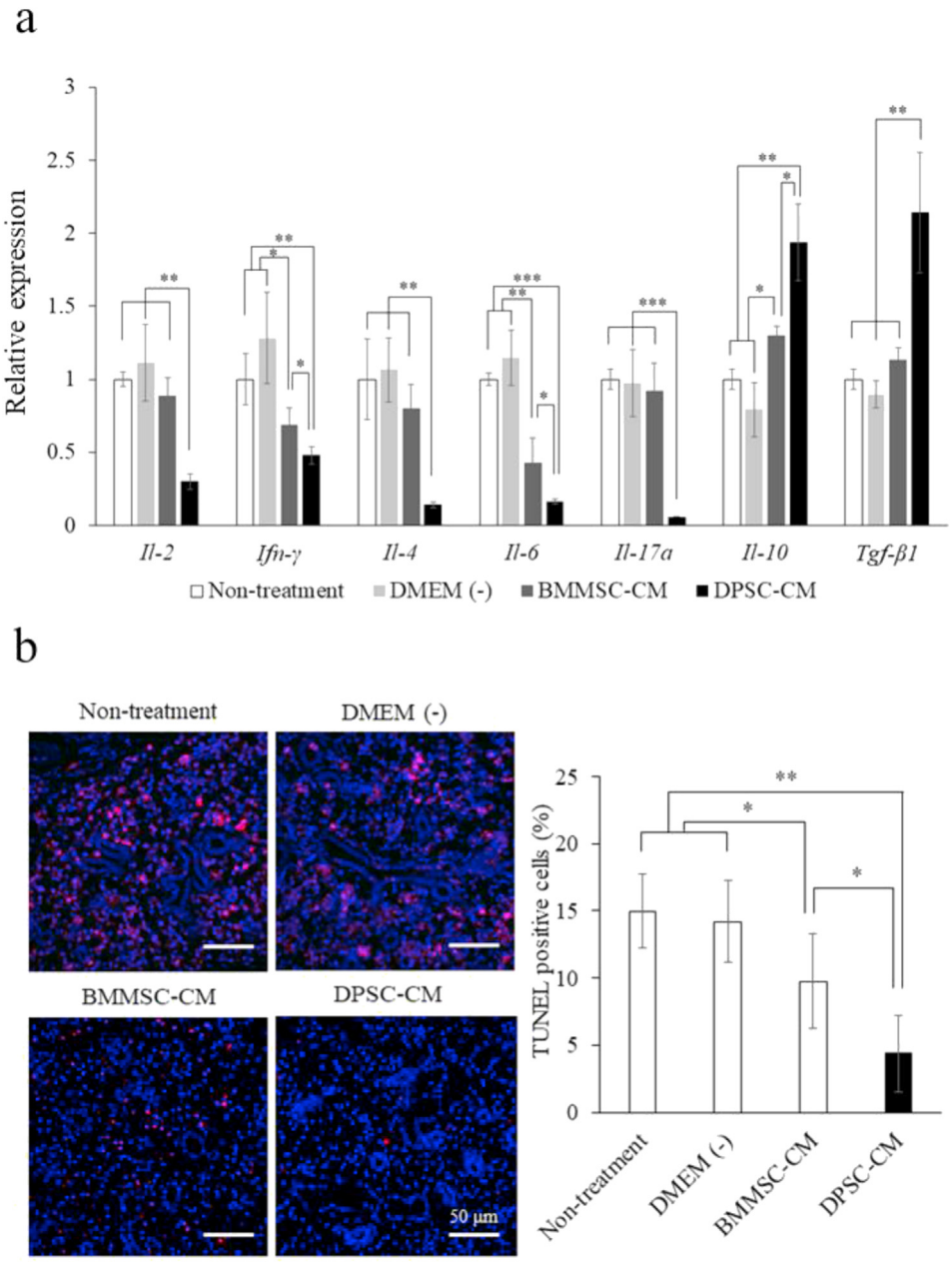


Fig. 3. mRNA expression levels of inflammatory and anti-inflammatory cytokines in the SMGs and TUNEL assays of the SMGs. (a) qRT-PCR analysis of the SMGs ($n = 6$ per group. $^{***}p < 0.00$, $^{**}p < 0.01$, $^{*}p < 0.05$). (b) TUNEL assays of the SMGs of the MRL/*lpr* mice reveal that the nuclei were stained with DAPI (blue) (Bars = 50 μm). Percentage of TUNEL-positive cells per total number of cells in the SMGs (right side). Data are representative of the mean ± standard deviation ($n = 6$, $^{**}p < 0.01$, $^{*}p < 0.05$).

body. As shown in Fig. 4, FOXP3-expressing cells, which were used as the marker of Treg cells, increased in the DPSC-CM-treated group. However, RORγ-expressing and GATA3-expressing cells which were used as markers of the type T helper 17 [Th17] and Th2 cells, respectively, decreased in the DPSC-CM-treated group than in the other groups. T-bet-expressing cells, which were used as the marker of Th1 cells, showed no changes in all groups.

4. Discussion

In this study, we evaluated the therapeutic effects of CM from DPSCs or BMMSCs in a secondary SS mouse model. DPSC-CM contains numerous anti-inflammatory factors (e.g., TGF-β1, IL-10, and IL-13) than BMMSC-CM (Fig. 1a and b).

The MRL/*lpr* strain is a well-established mouse model for SLE [21,22]. It is characterized by the inflammation of multiple tissues (e.g., skin, joints, glands, lungs, heart, and kidneys), massive

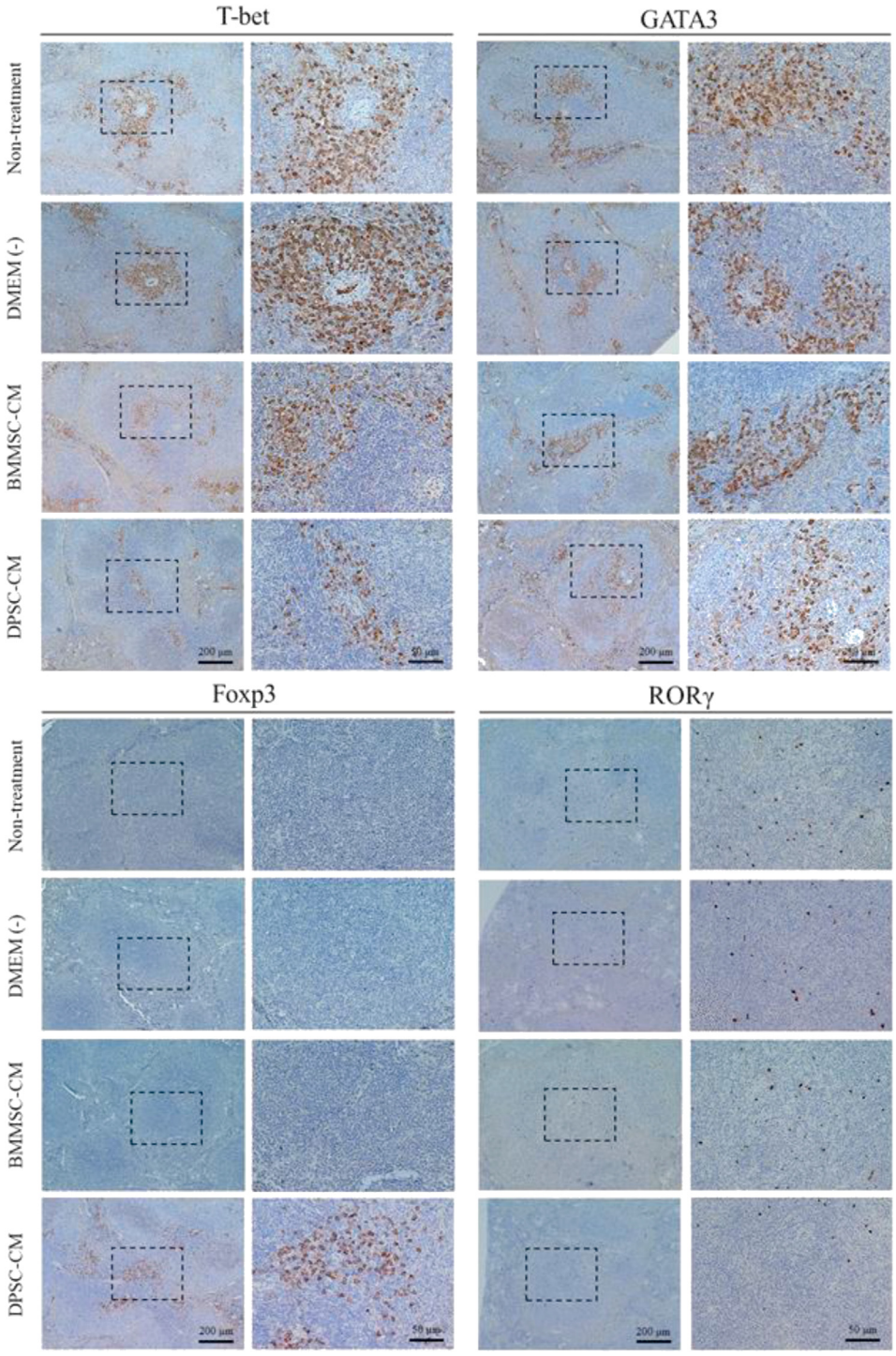


Fig. 4. Localization of each Th subset in the spleens of the MRL/*lpr* mice. Immunohistochemical staining for T-bet (Th1 cells), GATA3 (Th2 cells), Foxp3 (Treg cells), and RORγ (Th17 cells). Panels on the left ose on the right show the higher-magnification images (bars = 50 μm) of the areas surrounded by dotted lines.

lymphadenopathy, and splenomegaly, which progress in an age-dependent manner. Destructive mononuclear infiltrates in the lacrimal and SMGs of MRL/*lpr* mice are hallmarks of SS: we confirmed lymphocyte infiltration around the salivary duct cells in 12-week-old mice (Fig. 2b); the salivary flow rate declined at 12 weeks in MRL/*lpr* mice in the non-treatment and DMEM (−) groups than in the DPSC-CM group (Fig. 2d).

Extensive studies have been conducted to elucidate the immunomodulatory properties of MSCs; however, existing clinical trials of cell-based therapy are controversial, and their security is not guaranteed [23]. Many studies have determined that cell paracrine factors have functions similar to those of cells and the study of exosomes in the field of immunology is extensive [24–26]. Paracrine factors are better preserved, have lower risks of tumorigenesis and immune rejection, and can be used as alternative therapies for various immune diseases, comparable to cells [25].

A previous study reported that BMMSC-CM contains cytokines such as vascular endothelial growth factor, monocyte chemoattractant protein (MCP)-1, MCP-3, and hepatocyte growth factor. Many types of biomaterials and stem cell transplantation therapies have recently been proposed to enhance anti-inflammatory effects and functional recovery [19,27]. BMMSC acquisition is a difficult procedure; DPSC-CM is a more accessible resource, but its immunoregulation properties have not been fully studied.

Studies have shown that the Th17/Treg imbalance is a driving factor in the occurrence and development of immune diseases such as SLE and SS [28,29]. We investigated mRNA levels of inflammatory and anti-inflammatory cytokines, which are induced in the Th1, Th2, Treg, or Th17 cells of MRL/*lpr* mice SMGs. As shown in Fig. 3a, DPSC-CM administration decreased expression levels of *Ifn-γ*, *Il-6*, and *Il-17a* and increased those of *Il-10* and *Tgf-β1* in the SMGs, indicating Treg cell differentiation in the local environment. Furthermore, IFN-γ is primarily secreted by cytotoxic or Th1 T cells and natural killer cells [30]. One study reported that exogenous administration of IFN-γ with desiccating stress exposure increased epithelial apoptosis, indicating that IFN-γ promotes epithelial apoptosis through the extrinsic apoptosis pathway in SS [31]. We investigated and confirmed that DPSC-CM had an anti-apoptotic effect on the SMGs (Fig. 3b). We evaluated the kidneys of MRL/*lpr* mice, and the results suggested that DPSC-CM also improved the kidneys (e.g., creatinine level); decreased the expression levels of *Ifn-γ*, *Il-6*, and *Il-17a*; and increased the expression levels of *Il-10* and *Tgf-β1*, which can easily promote Treg cells in the kidneys (Supplementary Fig. 1).

Effector cytokines of Th17 and Treg cells have been increasingly recognized as key players in anaphylaxis, autoimmunity, and inflammation [32]. IL-10 produced by Treg cells resulted in amelioration of the severity of collagen-induced arthritis mice than wild-type mice [33]. Our experimental results confirmed that DPSC-CM induced Treg cells and suppressed Th1 and Th17 cells in splenic lymphocytes relative to other groups (Fig. 4).

5. Conclusions

This is the first study to report that DPSC-CM alleviates hyposalivation caused by SS by decreasing the number of inflammatory cytokines, regulating the local inflammatory microenvironment, and decreasing apoptosis in the SMGs. DPSC-CM shows promise as a novel option in cell-free therapy for various autoimmune diseases such as SLE and SS.

Declaration of competing interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2021.01.006.

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