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Stem cells from exfoliated deciduous teeth alleviate hyposalivation caused by Sjögren syndrome

Zhi-Hao Du[1] | Chong Ding[2] | Qian Zhang[2] | Yan Zhang[3] | Xi-Yuan Ge[2] | Sheng-Lin Li[2] | Guang-Yan Yu[1]

[1]Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing, China

[2]Center Laboratory, Peking University School and Hospital of Stomatology, Beijing, China

[3]Department of Physiology and Pathophysiology, Peking University School of Basic Medical Sciences, Beijing, China

Correspondence
Sheng-Lin Li, Center Laboratory, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Street, Haidian District, Beijing 100081, China.
Email: kqshlli@bjmu.edu.cn
and
Guang-Yan Yu, Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Street, Haidian District, Beijing 100081, China.
Email: gyyu@263.net

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Abstract

Objectives: To evaluate the effect of stem cells from exfoliated deciduous teeth on the hyposalivation caused by Sjögren syndrome (SS) and investigate the mechanism.

Methods: Stem cells were injected into the tail veins of non-obese diabetic mice, the animal model of SS. The saliva flow was measured after pilocarpine intraperitoneal injection. Apoptosis and autophagy were evaluated by TUNEL and Western blot. Lymphocyte proportions were detected by flow cytometer.

Results: Fluid secretion was decreased in 21-week-old mice. Stem cell treatment increased fluid secretion, alleviated inflammation in the submandibular glands and reduced inflammatory cytokine levels in the serum, submandibular glands and saliva. Stem cells decreased the apoptotic cell number and the expressions of ATG5 and Beclin-1 in the submandibular glands. Stem cells have no effect on other organs. Furthermore, the infused stem cells migrated to the spleen and liver, not the submandibular gland. Stem cells directed T cells towards Treg cells and suppressed Th1 and Tfh cells in spleen lymphocytes.

Conclusion: Stem cells from exfoliated deciduous teeth alleviate the hyposalivation caused by SS *via* decreasing the inflammatory cytokines, regulating the inflammatory microenvironment and decreasing the apoptosis and autophagy. The stem cells regulated in T-cell differentiation are involved in the immunomodulatory effects.

KEYWORDS
saliva, Sjögren syndrome, stem cell, stem cell therapy, submandibular gland

1 | INTRODUCTION

Sjögren syndrome (SS) is a chronic autoimmune disease primarily characterized by inflammation in salivary and lacrimal glands. The progressive damage to these glands induces hyposecretion and leads to the dryness of the mouth and eyes, respectively (Fox, 2005). However, the treatment of SS is challenging. Traditional pharmacological therapies, such as pilocarpine to stimulate residual acinar cells, are not aimed at the cause of the disease; therefore, they cannot repair the damaged gland and restore its secretion function (Ramos-Casals, Tzioufas, Stone, Siso, & Bosch, 2010).

Stem cell-based therapy offers a new strategy for hyposalivation. Adipose-derived stem cells (ADSCs) ameliorate salivary gland damage induced by radiation injury and improve salivary secretion (Li et al., 2015; Lim et al., 2013). Bone marrow mesenchymal stem cells (BMSCs) and umbilical cord mesenchymal stem cells (UCMSCs) could suppress inflammation and improve salivary gland function in a mouse model and in patients with SS, respectively (Xu et al., 2012). Interestingly,

Zhi-Hao Du and Chong Ding contributed equally to this work.

BMSCs were transdifferentiated into salivary gland acinar-like cells and injected into the radiation-damaged submandibular gland. The acinar-like cells could increase saliva production. More importantly, the saliva production in mice infused with acinar-like cells was increased more obviously compared with that in mice with BMSCs transplantation. It indicated that acinar-like cells had a better therapeutic effect for hyposalivation than BMSCs (Lin et al., 2011). In another study, tissue-specific and mesenchymal-like stem cells were isolated from human salivary glands. Transplantation of these stem cells into radiation-damaged rat salivary glands could rescue decreased secretion, recover acinar and duct structure, and decrease the number of apoptotic cells (Jeong et al., 2013). In addition, transplantation of salivary gland stem/progenitor cells could restore the secretory function of the irradiated gland (Pringle et al., 2016). These studies suggest that oral stem cells might exert a therapeutic effect on hyposalivation.

In postnatal dental tissue, several sources of stem cells have already been identified, such as dental pulp (Gronthos, Mankani, Brahim, Robey, & Shi, 2000), exfoliated deciduous teeth (Miura et al., 2003), periodontal ligament (Seo et al., 2004), dental follicle (Yao, Pan, Prpic, & Wise, 2008) and root apical papilla (Sonoyama et al., 2008). Isolated stem cells from exfoliated deciduous teeth are called stem cells from human exfoliated deciduous teeth (SHED) (Miura et al., 2003). Compared with other human stem cells, SHED represent an interesting stem cell source and are obtained using non-invasive techniques. To date, SHED have been used to treat diseases such as pulp–pulp dentin regeneration (Rosa, Zhang, Grande, & Nor, 2013), jaw regeneration (Jahanbin et al., 2015), nervous system diseases (Taghipour et al., 2012), immune system diseases (Ma et al., 2012), corneal injury (Gomes et al., 2010), liver injury (Yamaza et al., 2015), lung injury (Wakayama et al., 2015), kidney injury (Hattori et al., 2015) and diabetes (Kanafi et al., 2013). However, it is unknown whether SHED have effect on the hyposalivation caused by SS.

Therefore, the present study was designed to evaluate the effect of SHED on the hyposalivation caused by SS and, furthermore, to investigate the underlying mechanism.

2 | MATERIALS AND METHODS

2.1 | Cells culture

SHED were provided by ORAL STEM CELL BANK of Beijing, Tason Biotech Co., Ltd. The stem cells were cultured in MSC medium containing 10% foetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. Lymphocytes were isolated from the spleens of non-obese diabetic (NOD) mice and cultured in RPMI-1640 medium containing 10% foetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. All cell culture-related reagents were purchased from Gibco.

2.2 | SHED transplantation

Female NOD mice were got from Peking University Health Science Center. All experimental procedures were approved by the Peking University Institutional Review Board for the care and use of laboratory animals (approval number: LA2016316, date of approval: 2016.10.26). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. For transplantation, 1×10^6 SHED diluted in 200 μl of phosphate buffer saline (PBS) were infused into 7-week-old mice tail veins; 200 μl PBS was infused as control. The injection was performed once a week until the animals grew to 14 and 21 weeks.

2.3 | Measurement of stimulated saliva flow and biochemical composition analysis

Non-obese diabetic mice were under anaesthesia with chloral hydrate (0.4 g/kg body weight). Starting at 3 min after pilocarpine intraperitoneal injection (0.05 mg/100 g body weight), we used a micropipette to collect the whole saliva from the oral cavity for 10 min. The weight of the saliva was measured with a precision weighing balance (METTLER TOLEDO). After collecting the saliva, the α-amylase activity was detected, and the contents of sodium, potassium, chloride, calcium, magnesium and total protein were analysed by a Beckman AU5800 automatic biochemical analysis system (Beckman Coulter).

2.4 | Inflammatory cytokine expression analysis

The serum, saliva and submandibular gland tissues were collected from the NOD mice under anaesthesia. All inflammatory cytokine levels in the serum, saliva and submandibular gland lysates were measured by a Quantibody Mouse Th1/Th2/Th17 Array (QAM-TH17-1, RayBiotech, Inc.) in which 18 inflammatory cytokines were quantitatively examined: interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IL-17F, IL-21, IL-22, IL-23, IL-28, interferon-γ (IFN-γ), macrophage inflammatory protein-3 (MIP-3), transforming growth factor-β (TGF-β) and tumour necrosis factor-alpha (TNF-α).

2.5 | Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) staining

The fixed gland tissues were permeated with 20 μg/ml proteinase-K and then incubated with 3% H_2O_2. DNA fragmentation was detected using FragELTM DNA Fragmentation Detection Kit (Calbiochem) as previously described (Su et al., 2014). Labelled DNA was detected using a streptavidin–horseradish peroxidase conjugate, followed by reaction with diaminobenzidine. Sections were counterstained with methyl green. Apoptotic cells were displayed brown nucleus. Results were expressed as the number of positively staining cells per 4 mm^2 of glandular tissue.

2.6 | Western blot analysis

Protein (40 μg) from submandibular gland tissues was separated on a 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Then, the blocked membranes were incubated with

antibodies against ATG5 (1:1,000; Bioworld Technology), Beclin-1 (1:1,000; Cell Signaling Technology) or GAPDH (1:4,000; Huaxingbio), respectively. After probed with horseradish peroxidase-conjugated secondary antibodies (ZSGBBIO), the target proteins were detected using enhanced chemiluminescence reagent (Huaxingbio).

2.7 | SHED labelling and tracking

For stem cells tracking, the SHED were cultured with 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR; Invitrogen) for 2 hr. Next, the DiR-labelled SHED were transplanted into the NOD mice. The bioluminescence was measured with the IVIS Imaging System (Caliper Life Sciences) to monitor the location of SHED at 1, 2, 3 and 7 days after transplantation. On the 7th day, the submandibular gland, heart, lung, liver, pancreas, spleen, kidney and intestine tissues were collected and analysed.

In addition, green fluorescent protein (GFP) plasmid (Hanbio) was transferred into SHED as the manufacturer's instructions described. Next, the GFP-labelled SHED were infused into the NOD mice. On the 7th day, the submandibular glands were collected and make frozen section. Nuclei were labelled with 4,6-diamidino-2-phenylindole (DAPI) (ZSGBBIO). Images were captured by confocal microscope (LMS710, Carl Zeiss Microscopy).

2.8 | $CD4^+$ T Lymphocytes proportion analysis

Spleens lymphocytes were isolated from the NOD mice as the manufacturer's instructions described (Solarbio Life Science). For staining cytokine-producing cells, the lymphocytes (1×10^6) were stimulated with PMA/Ionomycin Mixture and BFA/Monensin Mixture (eBioscience) and then incubated with FITC-conjugated anti-CD4 antibody (BD Bioscience). For Th1, Th2 and Th17 cells staining, the lymphocytes were stained with PE-conjugated anti-IFN-γ and anti-IL-17, or APC-conjugated anti-IL-4 antibodies (eBioscience). For Tfh cells staining, the lymphocytes were stained with FITC-conjugated anti-CD4 antibody and APC-conjugated anti-CXCR5 antibody. For Treg cells staining, the lymphocytes were stained with FITC-conjugated anti-CD4 antibody and PE-conjugated anti-CD25 antibody, then fixation and permeabilization according to the manufacture's protocol, followed by staining with APC-conjugated anti-Foxp3 antibody (eBioscience). The lymphocytes were then harvested and analysed by a FACS Aria II Flow Cytometer (BD Bioscience).

2.9 | Statistical analysis

Data are shown as mean ± *SD*. Statistical analysis was performed by unpaired Student's *t* test between PBS- and SHED-transplanted groups or two-way analysis of variance (ANOVA) followed by Bonferroni's test among multiple groups using GraphPad Prism Software (version 6.00; GraphPad). $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | Evaluation of Sjögren syndrome-like characteristics in NOD mice

Non-obese diabetic mice are recognized as an animal model for SS (Humphreys-Beher, Hu, Nakagawa, Wang, & Purushotham, 1994). First, the secretory function of NOD mice was detected. After pilocarpine stimulation, the stimulated saliva flow rates were significantly decreased in 21-week-old mice, compared with those in 7- and 14-week-old mice ($p < 0.01$ and $p < 0.05$, respectively, Figure 1a). However, the activities of α-amylase in saliva were not different in 7-, 14- and 21-week-old mice (Figure 1b). Contents of sodium, potassium, chloride, calcium, magnesium and total protein were not changed in these three groups (Figure 1c–h).

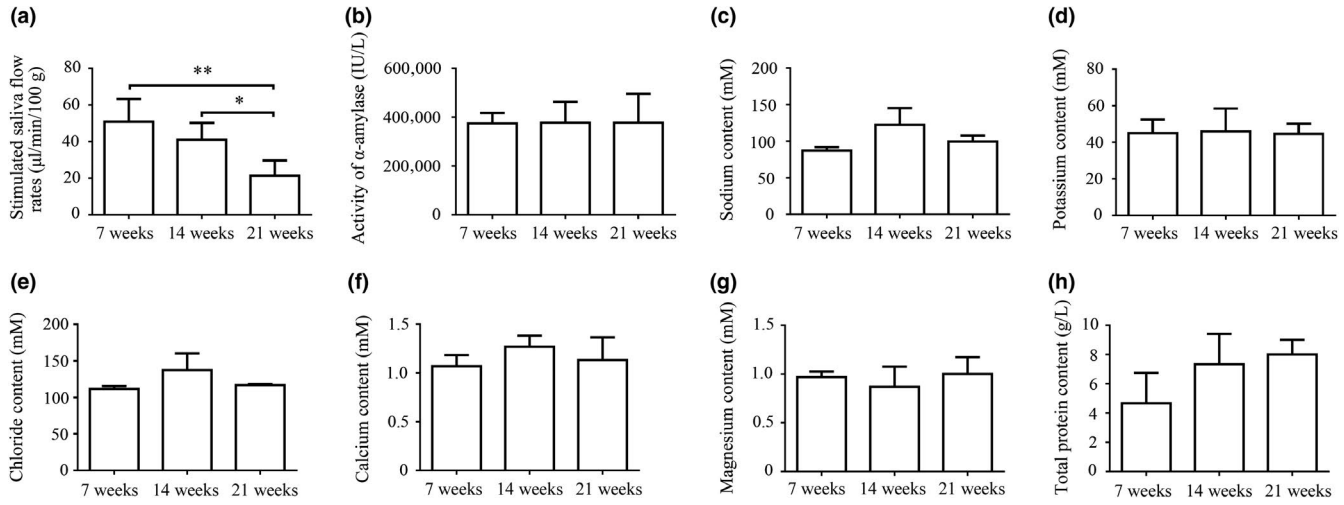

FIGURE 1 Saliva flow rate and biochemical composition analysis of NOD mice. (a) Stimulated saliva flow rate of 7-, 14- and 21-week-old NOD mice. (b) The α-amylase activity in the saliva of NOD mice. (c–h) The contents of sodium, potassium, chloride, calcium, magnesium and total protein in the saliva of NOD mice. Values represent the mean ± *SD* from 5 independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$

Next, the inflammation in the submandibular gland was assessed. Inflammatory cell foci were detected in 2 of 5 7-week-old mice and in all 14- and 21-week-old mice (Figure 2a–c). For further assess the inflammation clearly, we analysed the focus score and the ratio index. The focus scores were 1.02, 5.23 and 4.68, and the ratio index was 0.0005, 0.0211 and 0.0279 in 7-, 14- and 21-week-old mice, respectively. The focus scores and ratio index were both increased in 14- and 21-week-old mice compared with those in 7-week-old mice ($p < 0.01$, Figure 2d,e).

3.2 | Th1/Th2/Th17 cytokine profile in the serum, saliva and submandibular glands of NOD mice

$CD4^+$ Th1/Th2/Th17 cells are involved in SS pathogenesis (Mieliauskaite, Dumalakiene, Rugiene, & Mackiewicz, 2012; van Woerkom et al., 2005). Here, we fully detected the Th1/Th2/Th17 cytokine profiles in the serum, submandibular gland and saliva. Compared with those of in 7-week-old mice, the levels of IL-4, IL-6, IL-10, IL-12, IL-13, IL-17F, IL-23, MIP-3 and TGF-β (9/18) were increased in the serum of 14-week-old mice ($p < 0.05$). Compared with those in 7-week-old mice, all cytokines (18/18) were increased in the serum of 21-week-old mice ($p < 0.05$). The levels of IL-1β, IL-2, IL-5, IL-6, IL-13, IL-17, IL-17F, IL-21, IL-22, IL-23, IL-28, IFN-γ, MIP-3, TGF-β and TNF-α (15/18) were increased in the serum of 21-week-old mice, compared with those in 14-week-old mice ($p < 0.05$, Table 1).

Compared with those in 7-week-old mice, the levels of IL-1β, IL-6, IL-12, IL-21, IL-22 and TGF-β (6/18) were increased in the submandibular gland tissues of 14-week-old mice ($p < 0.05$). Compared with those in 7-week-old mice, the levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17F, IL-21, IL-22, IL-23, IL-28, IFN-γ, TGF-β and TNF-α (15/18) were increased in submandibular gland tissues of 21-week-old mice ($p < 0.05$). The levels of IL-2, IL-12, IL-13, IL-21, IL-23, IL-28 and IFN-γ (7/18) were increased in the submandibular gland tissues of 21-week-old NOD mice, compared with those in 14-week-old mice ($p < 0.05$, Table 2).

Compared with those in 7-week-old mice, the levels of IL-5, IL-13, IL-21 and MIP-3 (4/18) were increased in the saliva of 14-week-old mice ($p < 0.05$). The levels of IL-1β, IL-4, IL-6, IL-10, IL-13, IL-17, IL-21, IL-23, IL-28, IFN-γ, MIP-3, TGF-β and TNF-α (13/18) were increased in the saliva of 21-week-old mice ($p < 0.05$). The levels of IL-6, IL-10, IL-17, IL-23, IL-28, IFN-γ, TGF-β and TNF-α (8/18) were increased in the saliva of 21-week-old mice, compared with those of 14-week-old mice ($p < 0.05$, Table 3). These results suggest that the peripheral and local inflammatory cytokines are increased as the disease progressed.

3.3 | SHED transplantation alleviates the decrease in fluid secretion

To confirm whether SHED have an effect on hyposalivation induced by SS, we transplanted SHED into 7-week-old NOD mice once a week

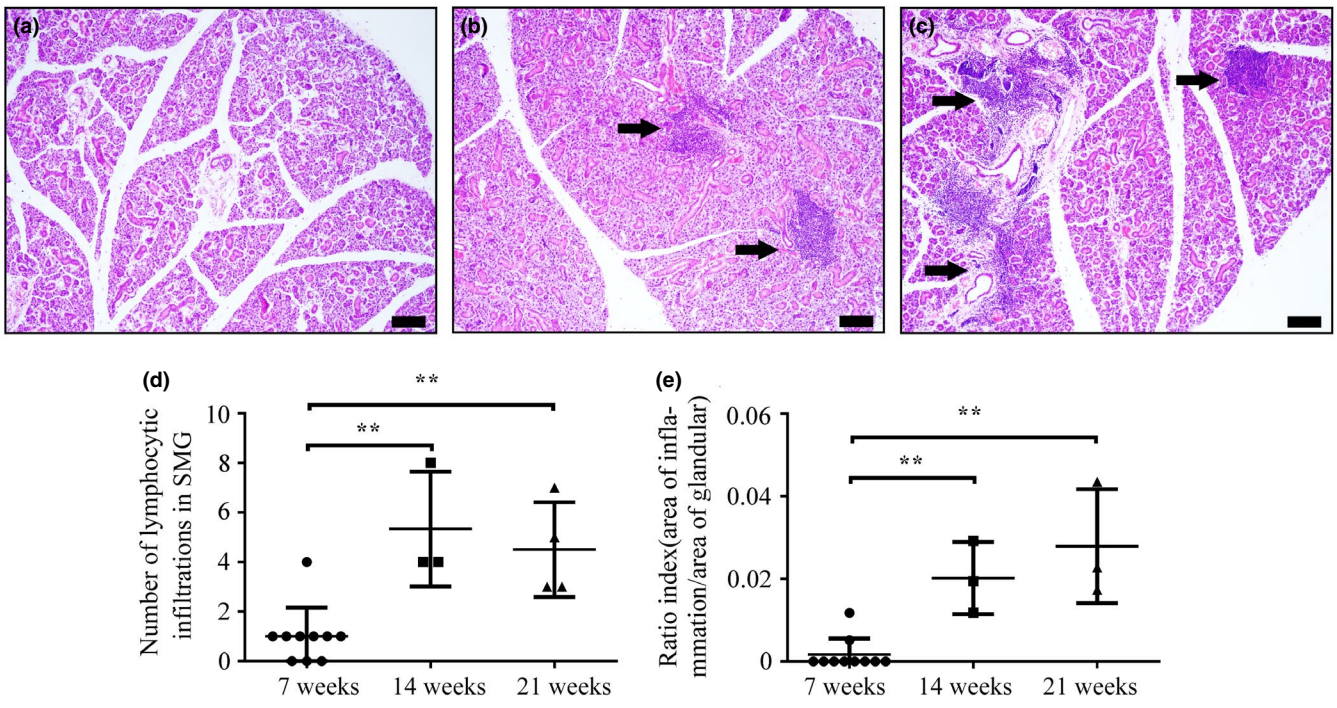

FIGURE 2 Evaluation of inflammatory infiltration in the submandibular glands of NOD mice. (a–c) Representative histological images of submandibular glands of 7-, 14- and 21-week-old NOD mice. Inflammatory cell foci are shown with arrows. Scale bar, 200 μm. The degree of inflammatory infiltration in the submandibular gland was evaluated by the focus score; (d) represents the number of foci comprising ≥50 mononuclear cells per 4 mm^2 of glandular tissue and the ratio index; (e) represents the ratio of the area of foci to the total area of glandular tissue. Values represent the mean ± *SD* from 5 independent experiments. $^{**}p < 0.01$

TABLE 1 The levels of Th1/Th2/Th17 cytokines in the serum in 7-, 14- and 21-week-old NOD mice

	7 weeks (pg/ml)	14 weeks (pg/ml)	21 weeks (pg/ml)
IL-1β	0.50 ± 0.71	1.47 ± 1.29	7.68 ± 1.05$^{\#\#}$
IL-2	16.61 ± 1.43	23.00 ± 5.88	206.08 ± 52.28$^{\#\#}$
IL-4	0.60 ± 0.15	0.90 ± 0.08*	1.18 ± 0.24*
IL-5	45.79 ± 13.26	60.65 ± 2.39	345.34 ± 30.42$^{\#\#}$
IL-6	17.15 ± 2.19	55.08 ± 19.22*	232.01 ± 34.65$^{\#\#}$
IL-10	39.38 ± 5.63	70.00 ± 8.28**	73.18 ± 11.99**
IL-12	12.92 ± 1.82	40.21 ± 7.99**	52.27 ± 9.72**
IL-13	148.50 ± 21.92	470.11 ± 181.89*	3,725.87 ± 166.84$^{\#\#}$
IL-17	10.20 ± 0.84	16.20 ± 11.34	78.65 ± 22.68$^{\#}$
IL-17F	20.51 ± 3.73	46.2 ± 1.29**	119.06 ± 23.04$^{\#\#}$
IL-21	0.14 ± 0.20	0.10 ± 0.15	0.75 ± 0.18*$^{\#\#}$
IL-22	0.00	0.05 ± 0.08	5.41 ± 0.44$^{\#\#}$
IL-23	353.47 ± 66.54	840.55 ± 41.27**	5,423.36 ± 1,010.35$^{\#\#}$
IL-28	23.08 ± 12.89	19.21 ± 2.36	170.03 ± 41.38$^{\#\#}$
IFN-γ	46.52 ± 27.21	90.29 ± 31.70	1,103.47 ± 95.69$^{\#\#}$
MIP-3	68.30 ± 14.26	181.66 ± 20.94**	476.41 ± 25.53$^{\#\#}$
TGF-β	348.34 ± 25.52	620.57 ± 75.18**	1996.96 ± 825.66*$^{\#}$
TNF-α	0.00	0.43 ± 0.40	2.65 ± 0.21$^{\#\#}$

Note: $n = 3$.
*$p < 0.05$ and **$p < 0.01$ compared with 7-week-old NOD mice. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with 14-week-old NOD mice.

TABLE 2 The levels of Th1/Th2/Th17 cytokines in submandibular gland tissues in 7-, 14- and 21-week-old NOD mice

	7 weeks (μg/mg)	14 weeks (μg/mg)	21 weeks (μg/mg)
IL-1β	21.36 ± 11.19	47.00 ± 3.54*	60.27 ± 14.97*
IL-2	16.02 ± 13.91	22.27 ± 20.92	142.11 ± 39.17**$^{\#\#}$
IL-4	0.77 ± 0.67	2.30 ± 2.35	5.00 ± 0.99**
IL-5	0.00	6.39 ± 11.07	0.00
IL-6	0.00	3.22 ± 0.79**	12.00 ± 6.20*
IL-10	204.57 ± 40.68	354.80 ± 125.83	525.11 ± 124.79*
IL-12	10.29 ± 6.68	73.02 ± 23.32*	223.74 ± 34.60**$^{\#\#}$
IL-13	636.24 ± 272.72	1,304.12 ± 451.71	3,184.40 ± 925.36*$^{\#}$
IL-17	0.00	0.00	0.00
IL-17F	10,355.16 ± 5,189.33	17,156.42 ± 10,184.27	31,505.69 ± 8,633.50*
IL-21	225.04 ± 61.37	550.73 ± 192.86*	1,304.92 ± 291.26**$^{\#}$
IL-22	317.33 ± 138.82	1,026.14 ± 377.85*	1875.18 ± 481.92**
IL-23	154.00 ± 63.50	183.50 ± 48.00	447.21 ± 113.50*$^{\#}$
IL-28	51.52 ± 5.62	52.78 ± 12.21	80.92 ± 4.90**$^{\#}$
IFN-γ	22.32 ± 38.66	42.41 ± 20.74	221.45 ± 105.56*$^{\#}$
MIP-3	2,494.88 ± 1837.17	2,585.37 ± 1846.11	18,393.28 ± 7,338.23
TGF-β	3,528.29 ± 295.17	5,074.53 ± 195.71**	4,977.61 ± 736.13*
TNF-α	0.00	6.00 ± 4.90	13.91 ± 6.50*

Note: $n = 3$.
*$p < 0.05$ and **$p < 0.01$ compared with 7-week-old NOD mice. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with 14-week-old NOD mice.

via the tail vein. As shown in Figure 3a, the stimulated saliva flow rates were decreased in 21-week-old mice, compared with that in 7- and 14-week-old mice ($p < 0.01$ and $p < 0.05$, respectively). The stimulated saliva flow rate of 14-week-old mice with SHED transplantation did not change compared with that in the PBS group and untreated groups. However, the stimulated saliva flow rate of

21-week-old mice with SHED transplantation was significantly increased, compared with the PBS and untreated groups ($p < 0.01$ and $p < 0.01$, respectively, Figure 3a). Furthermore, the flow rate in 21-week-old mice with SHED transplant was similar to that in 7-week-old mice. The α-amylase activity and the contents of sodium, potassium, chloride, calcium, magnesium and total protein did not differ between the PBS- and SHED-transplanted groups (Figure 3b–h). These results suggest that SHED transplantation is effective in alleviating the decrease in fluid secretion and maintaining normal secretory function.

3.4 | SHED transplantation inhibits the increase in inflammation of submandibular gland

The inflammation in the submandibular glands of 14-week-old mice with PBS and SHED transplantation is shown in Figure 4a,b. The focus scores and ratio index were not different between these two groups (Figure 4c,d). However, SHED alleviated inflammation in the submandibular glands of 21-week-old mice (Figure 4e,f). The focus scores and ratio index were both decreased in the SHED-transplanted group compared with those in the PBS group ($p < 0.01$ and $p < 0.01$, respectively, Figure 4g,h). These results suggest that SHED transplantation alleviates the inflammation of the submandibular glands.

3.5 | SHED transplantation decreases Th1/Th2/Th17 cytokine expression

Next, we evaluated the Th1/Th2/Th17 cytokine profile. Compared with those in the PBS group, the levels of IL-1β, IL-2, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17F, IL-28, IFN-γ, MIP-3, TGF-β and TNF-α (13/18) were decreased in the serum of 14-week-old mice transplanted with SHED ($p < 0.05$). The levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IL-17F, IL-21, IL-22, IL-23, IL-28, IFN-γ, MIP-3, TGF-β and TNF-α (17/18) were decreased in the serum of 21-week-old mice transplanted with SHED ($p < 0.05$, Table 4).

Compared with those in the PBS group, the levels of IL-1β, IL-5, IL-10, IL-13, IL-21, IL-22, IL-23, IL-28, IFN-γ, TGF-β and TNF-α (11/18) were decreased in the submandibular gland tissues of 14-week-old mice transplanted with SHED ($p < 0.05$). The levels of IL-1β, IL-2, IL-4, IL-10, IL-12, IL-13, IL-17F, IL-21, IL-22, IL-23, IL-28, IFN-γ, MIP-3, TGF-β and TNF-α (15/18) were decreased in the submandibular gland tissues of 21-week-old mice transplanted with SHED ($p < 0.05$, Table 5).

Compared with those in the PBS group, the levels of IL-2, IL-4, IL-5, IL-12, IL-13, IL-21, IL-23, IL-28, IFN-γ and TGF-β (10/18) were decreased in the submandibular gland tissues of 14-week-old mice transplanted with SHED ($p < 0.05$). The levels of IL-1β, IL-6, IL-10, IL-13, IL-17, IL-17F, IL-21, IL-23, IL-28, IFN-γ, MIP-3, TGF-β and TNF-α (13/18) were decreased in the submandibular gland tissues of 21-week-old NOD mice transplanted with SHED ($p < 0.05$, Table 6). These above results suggest that SHED reduce peripheral and local inflammatory cytokines level in NOD mice. Furthermore, this effect is more pronounced in the late stage of SS.

3.6 | SHED transplantation decreases apoptosis and autophagy in submandibular gland

Apoptosis and autophagy have been found to be upregulated in lesion tissue of SS patients, which are responsible for the impairment of

TABLE 3 The levels of Th1/Th2/Th17 cytokines in the saliva in 7-, 14- and 21-week-old NOD mice

	7 weeks (pg/ml)	14 weeks (pg/ml)	21 weeks (pg/ml)
IL-1β	1.06 ± 0.65	1.33 ± 0.52	3.27 ± 1.17*
IL-2	7.60 ± 1.23	7.82 ± 2.09	10.01 ± 3.54
IL-4	0.22 ± 0.10	0.31 ± 0.10	0.41 ± 0.05*
IL-5	3.18 ± 1.23	8.06 ± 1.58*	7.46 ± 2.48
IL-6	2.91 ± 0.54	3.26 ± 0.49	7.20 ± 1.98*#
IL-10	27.64 ± 7.40	35.62 ± 2.01	46.18 ± 2.79*##
IL-12	17.95 ± 2.31	20.18 ± 5.36	20.89 ± 4.34
IL-13	33.95 ± 2.32	45.06 ± 5.90*	49.21 ± 3.70**
IL-17	0.29 ± 0.26	0.17 ± 0.20	0.99 ± 0.31*#
IL-17F	0.00	0.00	0.42 ± 0.13
IL-21	0.97 ± 0.55	2.15 ± 0.49*	2.91 ± 0.95*
IL-22	0.40 ± 0.29	0.00	0.00
IL-23	17.10 ± 2.17	13.90 ± 4.27	25.84 ± 4.56*#
IL-28	2.56 ± 0.13	2.83 ± 0.49	8.58 ± 1.44**##
IFN-γ	1.28 ± 0.74	4.01 ± 1.60	14.72 ± 0.55**##
MIP-3	18.62 ± 8.59	115.09 ± 15.02**	98.28 ± 18.46**
TGF-β	273.20 ± 5.47	385.75 ± 71.15	962.97 ± 175.68**##
TNF-α	0.94 ± 1.62	1.53 ± 1.34	6.83 ± 1.74*#

Note: $n = 3$.
*$p < 0.05$ and **$p < 0.01$ compared with 7-week-old NOD mice. #$p < 0.05$ and ##$p < 0.01$ compared with 14-week-old NOD mice.

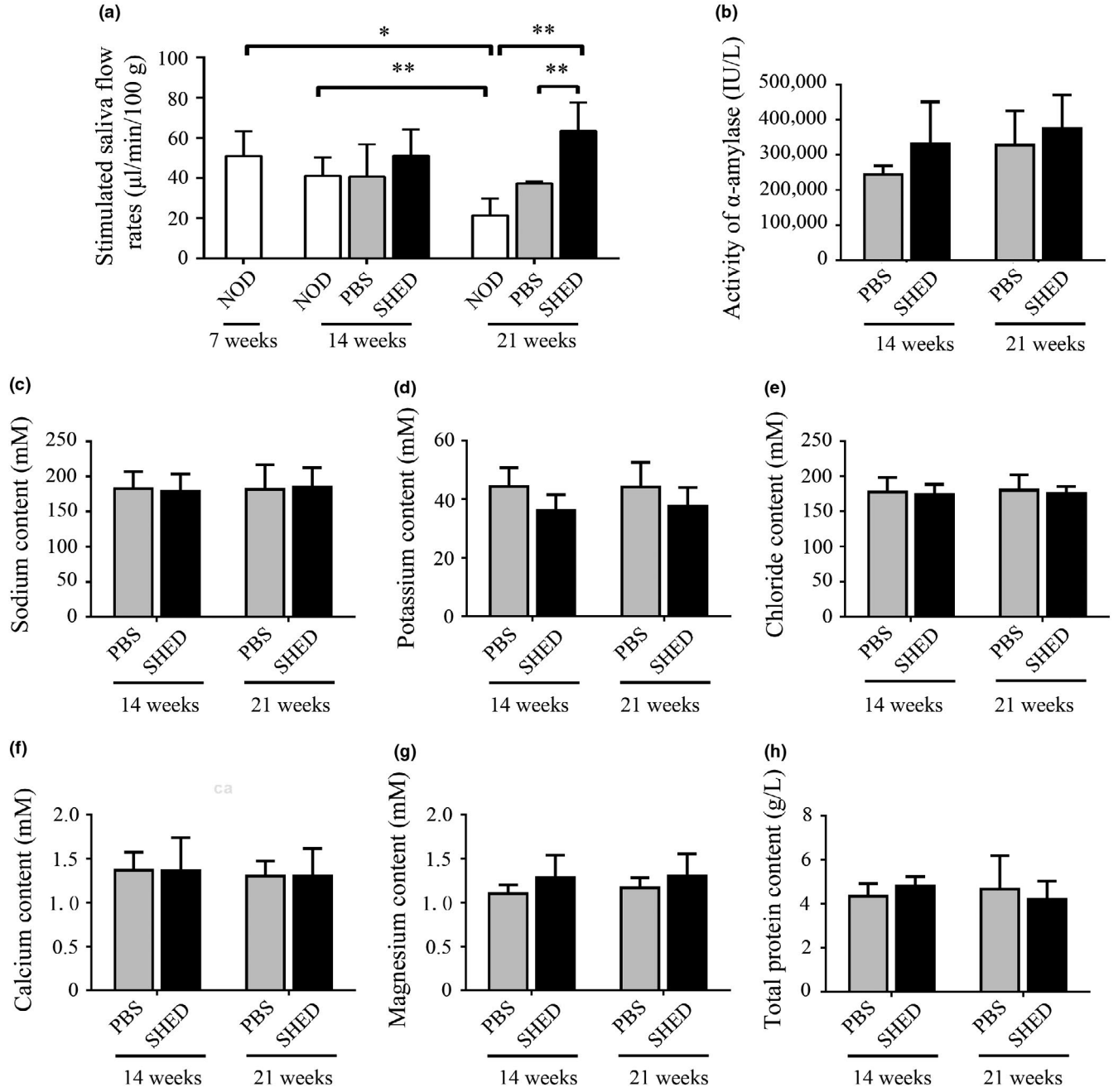

FIGURE 3 SHED transplantation restored the secretory function of NOD mice. Seven-week-old NOD mice were treated with PBS or SHED (1×10^6 cells) *via* the tail vein, and the stimulated saliva flow rates were measured and analysed at 14 and 21 weeks. (a) Stimulated saliva flow rate; (b) the α-amylase activity; (c) the sodium content; (d) the potassium content; (e) the chloride content; (f) the calcium content; (g) the magnesium content; (h) the total protein content. Values represent the mean ± *SD* from 5 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$

gland secretory function (Byun, Lee, Shin, & Chung, 2017; Manganelli & Fietta, 2003). Here, compared with this in 7- and 14-week-old NOD mice, the numbers of apoptotic cells were increased in the submandibular glands of 14- and 21-week-old NOD mice. SHED treatment decreased the number of apoptotic cells (Figure 5a,b). In addition, we also evaluated autophagy in the submandibular glands. We detected the expressions of ATG5 and Beclin-1, the specific markers of autophagy (Huang et al., 2014; Ma et al., 2015). Compared with those of 7-week-old NOD mice, the expressions of ATG5 and Beclin-1 were increased in 21-week-old NOD mice, which suggested that autophagy is increased as the disease progresses. Furthermore, SHED treatment decreased the expressions of ATG5 and Beclin-1 (Figure 5c–e). These above results suggest that SHED transplantation decreases apoptosis and autophagy in the submandibular glands.

3.7 | Evaluation of the safety of SHED transplantation

To evaluate the safety of SHED transplantation *via* the tail vein, we collected the parotid gland, heart, lung, liver, pancreas, spleen,

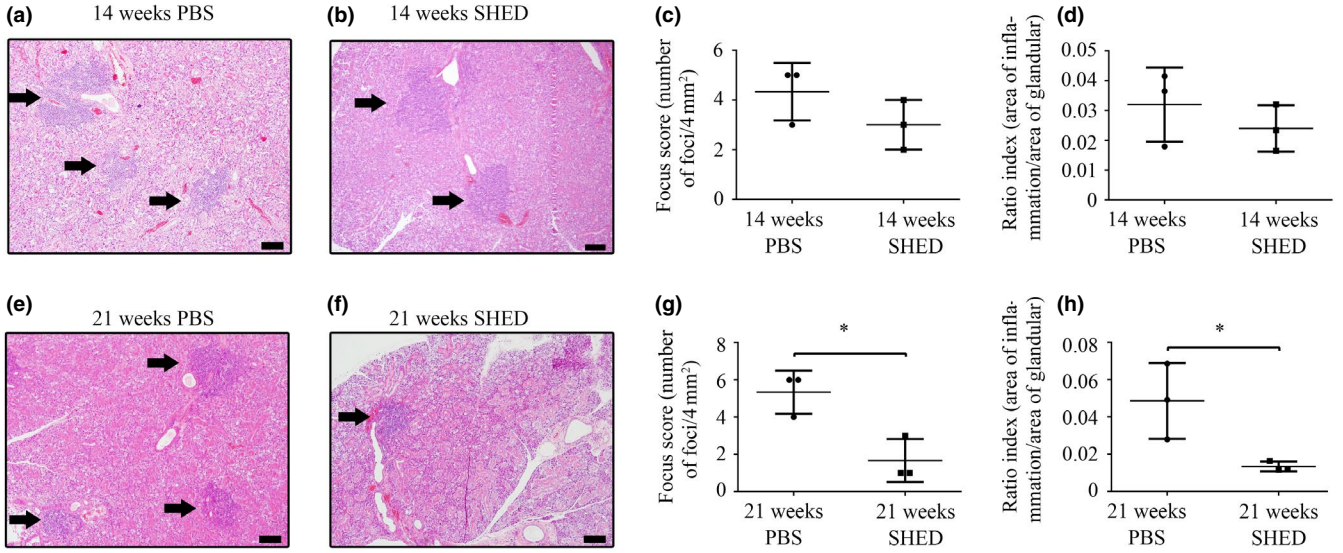

FIGURE 4 SHED transplantation decreased inflammatory infiltration in the submandibular gland of NOD mice. (a and b) Representative histological images of submandibular glands in 14-week-old NOD mice with or without SHED transplantation. Inflammatory cell foci are shown with arrows. Scale bar, 200 μm. The degree of inflammatory infiltration in the submandibular gland of NOD mice was evaluated by the focus score (c) and the ratio index (d). (e and f) Representative histological images of submandibular glands in 21-week-old NOD mice with or without SHED transplantation. Inflammatory cell foci are shown with arrows. Scale bar, 200 μm. The focus score (g) and the ratio index (h) were evaluated. Values represent the mean ± *SD* from 5 independent experiments. $^{*}p < 0.05$

kidney and intestine tissues. As shown in Figure S1a–h, the histological morphology of these organs did not change between these two groups. We also collected the faecal samples and analysed the faecal genomic DNA. As shown in Figure S2a–d, alpha diversity analysis of bacterial community richness (Chao1, Observed OTUs and PD whole tree) and the Shannon index showed that there

TABLE 4 The levels of Th1/Th2/Th17 cytokines in the serum of NOD mice in PBS- and SHED-transplanted groups

	14-week-old NOD mice		21-week-old NOD mice	
	PBS (pg/ml, *n* = 3)	SHED (pg/ml, *n* = 12)	PBS (pg/ml, *n* = 3)	SHED (pg/ml, *n* = 6)
IL-1β	2.41 ± 1.62	0.50 ± 0.88*	7.07 ± 2.22	0.46 ± 0.89**
IL-2	27.62 ± 2.41	19.01 ± 3.22**	250.18 ± 27.86	58.12 ± 33.23**
IL-4	0.80 ± 0.31	0.45 ± 0.27	1.48 ± 0.32	0.68 ± 0.35*
IL-5	89.67 ± 16.83	49.92 ± 19.93**	310.47 ± 62.97	37.81 ± 14.25**
IL-6	54.83 ± 13.51	17.02 ± 9.44**	215.87 ± 11.52	65.83 ± 35.88**
IL-10	74.47 ± 9.18	40.25 ± 10.36**	78.80 ± 22.70	49.15 ± 15.95
IL-12	46.58 ± 7.06	23.23 ± 11.90**	45.39 ± 13.73	17.13 ± 6.81**
IL-13	466.29 ± 192.86	145.55 ± 23.50**	3,662.71 ± 212.76	118.50 ± 47.79**
IL-17	17.52 ± 8.90	10.39 ± 10.21	70.16 ± 19.70	16.66 ± 8.89**
IL-17F	52.81 ± 5.94	38.42 ± 3.27**	109.87 ± 36.45	53.98 ± 23.49*
IL-21	0.16 ± 011	0.13 ± 0.13	0.82 ± 0.25	0.20 ± 0.24**
IL-22	0.05 ± 0.61	0.05 ± 0.00	6.05 ± 1.10	0.35 ± 0.05**
IL-23	737.65 ± 295.68	519.50 ± 147.04	4,813.92 ± 1652.27	216.48 ± 56.28**
IL-28	27.14 ± 8.72	17.28 ± 5.17*	160.07 ± 28.63	18.84 ± 4.37**
IFN-γ	98.74 ± 23.40	36.70 ± 32.89**	1,201.81 ± 180.34	57.78 ± 44.38**
MIP-3	200.41 ± 15.23	152.36 ± 20.69**	489.52 ± 5.25	152.21 ± 10.56**
TGF-β	563.53 ± 83.61	261.14 ± 56.09**	2,282.78 ± 925.93	146.21 ± 71.34**
TNF-α	0.22 ± 0.39	0.00**	0.40 ± 0.81	0.00**

Note: *n* = 3.
*$p < 0.05$ and **$p < 0.01$ compared with PBS-transplanted NOD mice.

TABLE 5 The levels of Th1/Th2/Th17 cytokines in the submandibular gland of NOD mice in PBS- and SHED-transplanted groups

	14-week-old NOD mice		21-week-old NOD mice	
	PBS (μg/mg, *n* = 3)	SHED (μg/mg, *n* = 12)	PBS (μg/mg, *n* = 3)	SHED (μg/mg, *n* = 6)
IL-1β	44.90 ± 30.31	15.33 ± 7.50**	79.80 ± 26.47	5.95 ± 3.42**
IL-2	25.76 ± 15.76	16.37 ± 10.89	147.16 ± 18.49	13.61 ± 10.65**
IL-4	1.60 ± 1.90	0.33 ± 1.01	3.89 ± 2.05	0.63 ± 1.33*
IL-5	7.88 ± 5.99	1.09 ± 1.27**	1.62 ± 2.80	0.00
IL-6	4.00 ± 1.72	2.62 ± 2.43	10.87 ± 7.47	4.40 ± 1.54
IL-10	417.14 ± 102.10	120.94 ± 150.52**	584.36 ± 117.48	130.88 ± 125.40**
IL-12	89.71 ± 52.72	66.47 ± 29.98	251.92 ± 62.88	67.22 ± 63.77**
IL-13	1,353.14 ± 568.47	464.95 ± 184.33**	2,892.58 ± 383.99	232.56 ± 136.65**
IL-17	0.00	0.00	0.00	0.00
IL-17F	20,157.52 ± 6,578.21	15,954.63 ± 9,813.45	35,783.28 ± 9,243.54	12,587.63 ± 8,416.52**
IL-21	487.87 ± 242.24	120.15 ± 16.94**	1,243.92 ± 93.70	142.18 ± 156.80**
IL-22	1,231.075 ± 105.72	161.69 ± 210.10**	1922.57 ± 166.53	121.62 ± 169.61**
IL-23	278.89 ± 61.84	62.39 ± 149.91**	380.63 ± 132.40	0.00**
IL-28	60.52 ± 15.07	36.90 ± 7.44**	61.36 ± 19.67	1.19 ± 2.66**
IFN-γ	56.53 ± 31.82	0.44 ± 1.52**	193.34 ± 39.17	0.33 ± 0.75**
MIP-3	2,742.36 ± 2,166.33	2,269.21 ± 1,353.98	12,983.56 ± 6,523.84	6,523.14 ± 1,128.36*
TGF-β	5,630.72 ± 255.79	3,050.00 ± 159.54**	5,110.89 ± 446.47	3,150.64 ± 521.09**
TNF-α	4.54 ± 2.16	2.07 ± 1.09*	10.97 ± 1.50	0.00**

*$p < 0.05$ and
**$p < 0.01$ compared with PBS-transplanted NOD mice.

were not different between the PBS- and SHED-transplanted groups. Beta-diversity analysis based on weighted UniFrac distance indicates that the structure and composition were not different between the two groups (Figure S2e,f). These results show that SHED transplantation has no effect on the histological morphology of other organs and the faecal microbiota structure and composition.

3.8 | SHED homing in vivo

To further investigate the underlying mechanisms by which SHED alleviate the decrease in fluid secretion, we labelled SHED with DiR and GFP and infused them into 14-week-old mice. As shown in Figure 6a, the DiR-labelled SHED were distributed in the abdomen 1 hr after transplantation, and their positive intensities increased gradually from 1 hr to 7 days (Figure 6a–d). To confirm the exact targeting of SHED, we collected and scanned the submandibular gland, heart, lung, liver, spleen, kidney, pancreas and intestine tissues on the 7th day. As shown in Figure 6e, the positive intensities were observed mainly in the liver and spleen. However, there was no signal in the submandibular gland, heart, lung, kidney, pancreas, stomach and intestine tissues. In addition, the GFP-labelled SHED was transplanted and examined on the 7th day. As shown in Figure 6f, there was no signal in the submandibular gland.

3.9 | SHED regulate $CD4^+$ T-cell differentiation

We next investigated whether and how SHED directed $CD4^+$ T-cell differentiation in spleen tissues. First, we detected the histology and morphology of spleen tissues and found there were no difference among 7-, 14- and 21-week-old mice (Figure S3a–c).

Then, we isolated spleen lymphocytes from the mice and co-cultured them with or without SHED. Compared with those in 7-week-old mice, the $CD4^+$ T-cell proportions were not changed in 14-week-old mice. SHED had no effect on $CD4^+$ T-cell proportions (Figure 7a,b). A predominance of Th1 cells was infiltrated in diseased salivary gland (Kallenberg, Vissink, Kroese, Abdulahad, & Bootsma, 2011; Mariette & Gottenberg, 2010). Here, the Th1 (IFN-γ positive) cell proportion was increased in spleen lymphocytes of 14-week-old mice ($p < 0.05$, Figure 7c,d). SHED significantly decreased the percentage of Th1 cell proportion ($p < 0.01$, Figure 7c,d). However, the Th2 (IL-4 positive) and Th17 (IL-17 positive) cell proportions were not different among 7-, 14- and 21-week-old mice co-cultured with SHED (Figure 7c,e,f,g). T regulatory (Treg) cells are reported diminished in the diseased salivary gland of SS patients (Li et al., 2007). Here, compared with that in the untreated group, SHED increased the percentage of Treg cell proportion (Figure 7h,i). T follicular helper T (Tfh) cells, a subset of $CD4^+$ helper T cells, could regulate the development of antigen-specific B-cell immunity (Fazilleau, Mark, McHeyzer-Williams, & McHeyzer-Williams, 2009). Compared

TABLE 6 The levels of Th1/Th2/Th17 cytokines in the saliva of NOD mice in PBS- and SHED-transplanted groups

	14-week-old NOD mice		21-week-old NOD mice	
	PBS (pg/ml, *n* = 3)	SHED (pg/ml, *n* = 12)	PBS (pg/ml, *n* = 3)	SHED (pg/ml, *n* = 6)
IL-1β	1.09 ± 0.18	0.86 ± 0.59	2.94 ± 0.59	0.51 ± 0.29**
IL-2	9.20 ± 0.11	5.62 ± 1.45**	12.13 ± 1.96	7.96 ± 4.57
IL-4	0.41 ± 0.39	0.02 ± 0.01**	0.63 ± 0.31	0.26 ± 0.23
IL-5	10.35 ± 3.47	5.66 ± 2.77*	8.79 ± 2.68	5.27 ± 3.22
IL-6	3.86 ± 0.42	3.47 ± 0.93	7.97 ± 0.08	1.85 ± 0.35**
IL-10	38.64 ± 5.69	28.64 ± 7.69	57.18 ± 10.81	18.92 ± 13.01**
IL-12	19.45 ± 5.48	15.06 ± 1.94*	21.28 ± 6.57	12.28 ± 5.01
IL-13	39.48 ± 10.59	3.46 ± 0.47**	54.75 ± 12.89	0.00**
IL-17	0.55 ± 0.33	0.13 ± 0.18	1.35 ± 0.58	0.29 ± 0.20**
IL-17F	0.00	0.00	0.64 ± 0.21	0.12 ± 0.10**
IL-21	2.35 ± 0.63	0.20 ± 0.17**	3.16 ± 0.80	0.00**
IL-22	0.10 ± 0.17	0.12 ± 0.40	0.12 ± 0.21	0.00
IL-23	15.48 ± 5.46	9.25 ± 3.48*	38.59 ± 15.05	9.83 ± 5.60**
IL-28	2.95 ± 0.54	4.77 ± 0.95**	10.49 ± 2.27	2.35 ± 0.89**
IFN-γ	5.57 ± 1.13	3.87 ± 0.95*	11.18 ± 3.54	0.00**
MIP-3	89.98 ± 28.14	77.51 ± 10.40	102.98 ± 12.38	57.62 ± 15.24**
TGF-β	315.84 ± 71.15	117.95 ± 14.60**	884.92 ± 142.73	27.65 ± 15.96**
TNF-α	1.69 ± 0.93	2.94 ± 1.92	8.37 ± 2.14	0.48 ± 1.30**

*$p < 0.05$ and
**$p < 0.01$ compared with PBS-transplanted NOD mice.

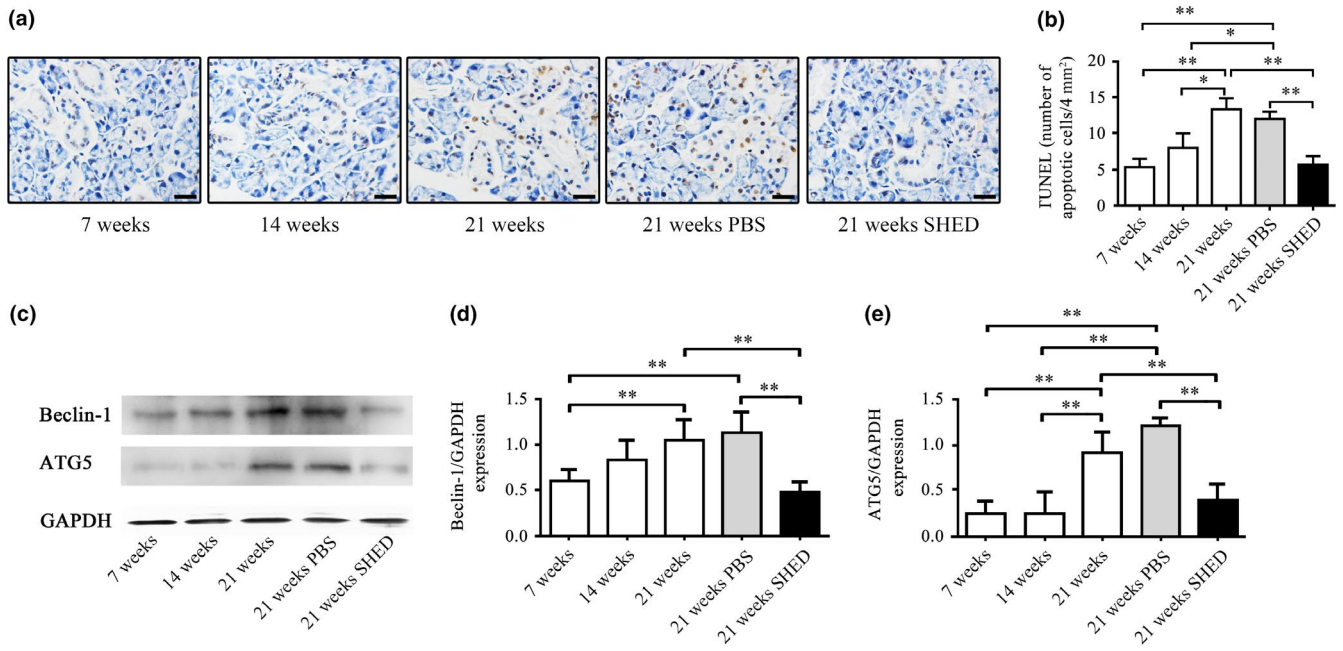

FIGURE 5 SHED transplantation decreased the apoptosis and autophagy in the submandibular glands of NOD mice. (a) Representative images of TUNEL staining in the submandibular glands of NOD mice. (b) Number of apoptotic cells in 4 mm^2 of glandular tissue was evaluated by TUNEL staining. Scale bar, 20 μm. (c) The expressions of ATG5 and Beclin-1 in the submandibular glands of NOD mice. (d and e) Quantitative analysis of ATG5 and Beclin-1 expression normalized to GAPDH. Values represent the mean ± *SD* from 3 independent experiments. *$p < 0.05$ and **$p < 0.01$

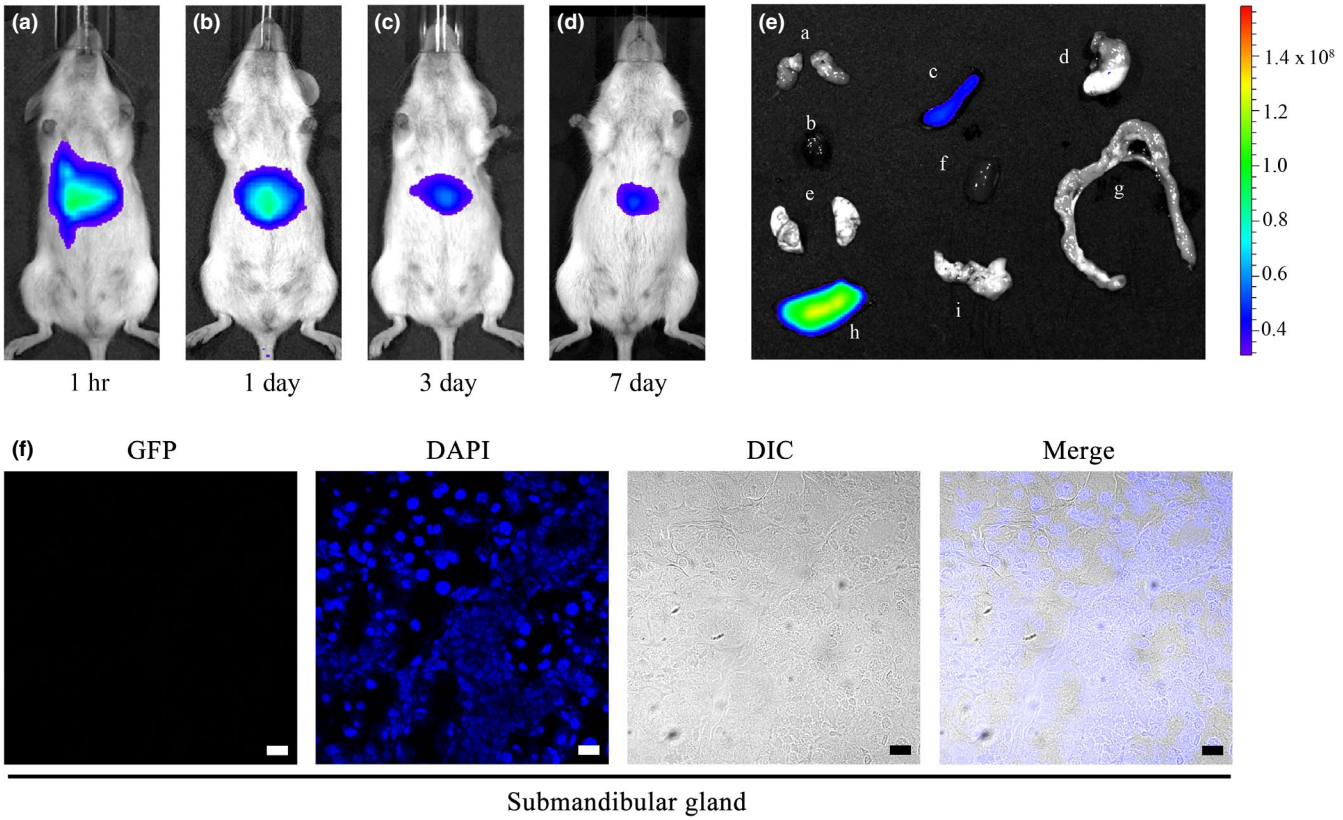

FIGURE 6 The homing of SHED in vivo. SHED were labelled with DiR and GFP and then transplanted into 14-week-old NOD mice. The bioluminescence was detected. Representative images of 14-week-old NOD mice after injection of CiR-labelled SHED. (a) 1 hr after injection; (b) 1 day after injection; (c) 3 days after injection; (d) 7 days after injection; (e) other organs were collected and detected on the 7th day. a: submandibular gland, b: heart, c: spleen, d: stomach, e: lung, f: kidney, h: liver, g: intestines, i: pancreas tissues. (f) Representative images of submandibular gland on the 7th day after injection of GFP-labelled SHED. Nuclei were stained with DAPI (blue)

with those in 7-week-old mice, the Tfh cell proportion was increased in 14-week-old mice. SHED decreased the percentage of Tfh cell proportion (Figure 7j,k). These results suggest that SHED direct T cells towards Treg cells and suppressed Th1 and Tfh cells in spleen lymphocytes.

4 | DISCUSSION

In this study, we confirmed that SHED alleviated the decrease in fluid secretion of NOD mice. After SHED treatment, the peripheral and local inflammatory cytokines were decreased. SHED stopped the inflammation, apoptosis and autophagy of submandibular gland from worsening. Finally, normal secretory function was maintained. The infused SHED migrated to spleen. SHED decreased Th1 and Tfh cells, and increased Treg cell differentiation in the spleen might be involved in the immunomodulatory effects.

Non-obese diabetic mice, the most commonly used animal model of SS, have chronic lymphocytic infiltration of endocrine and exocrine glands (Humphreys-Beher et al., 1994). The inflammation in the salivary glands was developed in 7- to 8-week-old mice (initial stage). Severe inflammatory lesions appeared in mice at the ages of 12–16 weeks (the early stage of clinical phase) and 20–24 weeks (the late stage of clinical phase), respectively (Yamano, Atkinson, Baum, & Fox," 1999). Corresponding to inflammatory infiltrates, the beginning of a slight decline in saliva flow rate was observed in mice between 8 and 12 weeks of age. Significantly decreased saliva secretion appeared in mice, beginning between 16 and 24 weeks of age (Yamano Atkinson, Baum, & Fox, 1999). In our study, comparing with that in 7-week-old mice, the saliva flow rate declined in 14-week-old mice and significantly decreased in 21-week-old mice. Inflammatory lesions appeared in 7-week-old mice and were significantly present in 14- and 21-week-old mice. The SS-like phenotype in NOD mice was consistent with our previous study (Su et al., 2014).

The infiltrated lymphocytes in the salivary glands of SS are mainly $CD4^+$ T cells (Skopouli et al., 1991). Both Th1 and Th2 cytokine levels increased in salivary glands of SS patients (Fox & Speight, 1996; Fox, Kang, Ando, Abrams, & Pisa, 1994; Ohyama et al., 1996; Sun, Emmert-Buck, & Fox, 1998). In detail, the elevated levels of the IL-1β and TNF-α (proinflammatory cytokines), IL-2 and IFN-γ (Th1 cytokines), and IL-10 (Th2 cytokine) have been reported. In NOD mice, IL-1β, IL-2, IL-10, TNF-a and IFN-γ levels in the salivary glands are increased, which are similar to that in SS patients (Robinson et al., 1998). IL-17 secreted by Th17 cell also participates in SS progression. IL-17 expression is increased in the plasma and labial salivary glands

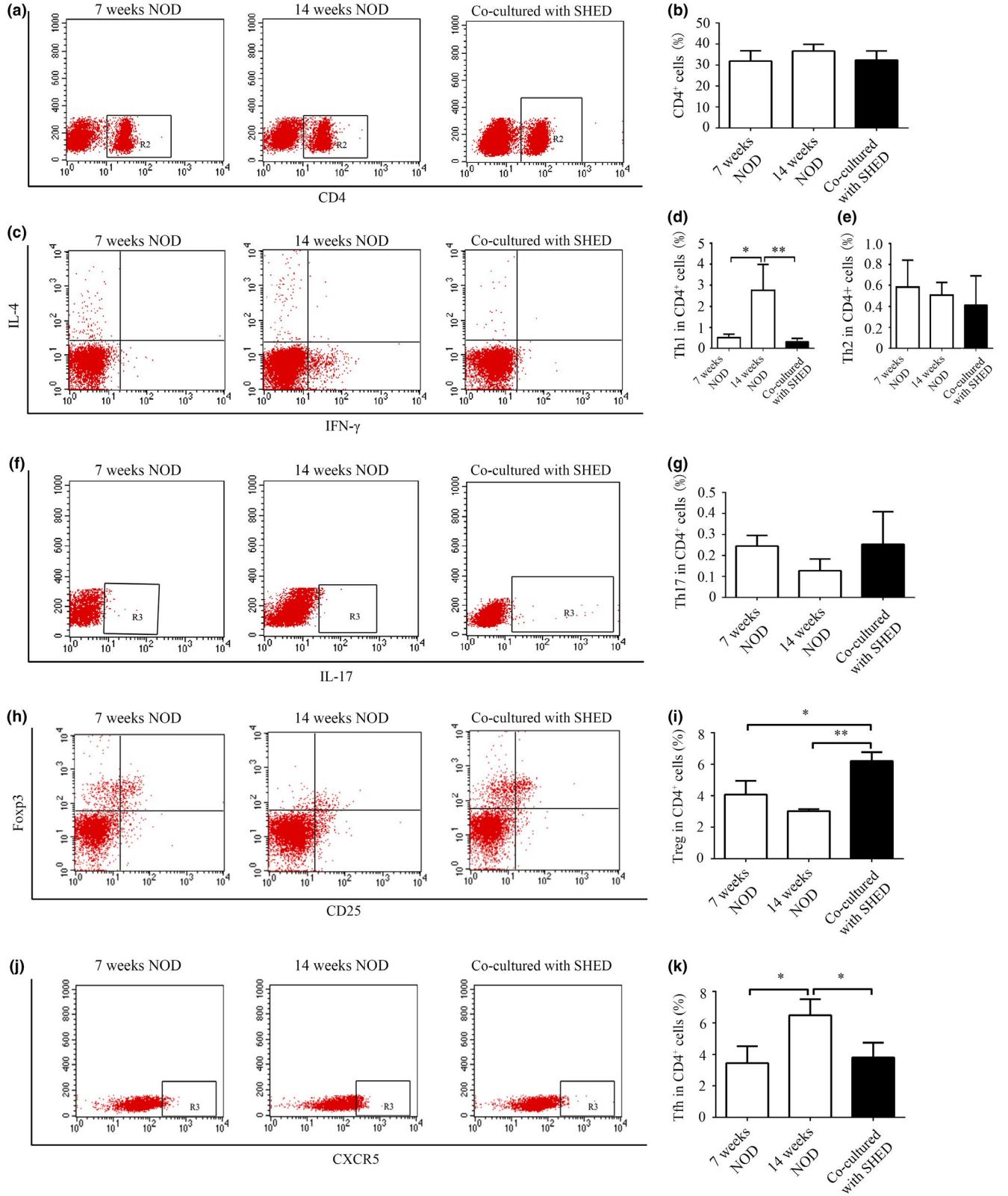

FIGURE 7 SHED treatment regulates $CD4^+$ T-cell response. Lymphocytes were isolated from the spleen of 7- and 14-week-old NOD mice with or without SHED co-culture. The proportions of $CD4^+$ T, Th1, Th2, Th17, Treg and Tfh cells were detected. (a and b) The proportion of $CD4^+$ T cells in lymphocytes. (c, d and e) The percentage of Th1 cells (IFN-γ positive) and Th2 cells (IL-4 positive) in $CD4^+$ T cells. (f and g) The percentage of Th17 cells (IL-17 positive) in $CD4^+$ T cells. (h and i) The percentage of Treg cells (Foxp3 and CD25 positive) in $CD4^+$ T cells. (j and k) The percentage of Tfh cells (CD4 and CXCR5 positive) in $CD4^+$ T cells. Values represent the mean ± *SD* from 5 independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$

of SS patients (Mieliauskaite et al., 2012). Furthermore, our previous study indicated that infiltrated lymphocyte-derived IL-17 could impair tight junction barrier of glandular epithelial cells to alter the secretory function of the submandibular gland in NOD mice (Zhang et al., 2016). However, these studies focus on either the salivary gland (local) or the blood (peripheral) samples in term of some of the cytokines. Here, to fully identify inflammatory cytokines involved in SS, we examined the Th1/Th2/Th17 cytokine profile in the serum, submandibular gland tissues and saliva of NOD mice. We found that more Th1/Th2/Th17 cytokines were increased in the serum, submandibular gland tissues and saliva as the disease progressed. These results were consistent with the decreased secretory function and increased inflammation in the submandibular gland.

Infusion of BMSCs into NOD mice for 2 weeks could alleviate the inflammatory responses in submandibular glands and restore their secretion function. These effects could work in both 6- and 16-week-old NOD mice. These results suggest that BMSCs exert protective and therapeutic effects on SS in a short time (Xu et al., 2012). In this study, we transplanted SHED into 7-week-old mice and conducted a long-term follow-up. We found that the secretion was not decreased in 14-week-old mice, compared with that in 7-week-old mice. SHED did not affect the secretory function. However, the fluid secretion was significantly decreased in untreated 21-week-old NOD mice, while it was not decreased in the SHED-transplanted group. The secretory function did not decrease with age. These results suggest that SHED transplantation protects the salivary gland and maintains normal secretory function.

Furthermore, lymphocyte infiltration in the submandibular gland was increased in 14-week-old NOD mice. SHED did not decrease the lymphocyte infiltration. However, the levels of some Th1/Th2/Th17 cytokines in the serum, submandibular gland tissues and saliva were decreased. In 21 weeks, SHED increased the secretory function and decreased the inflammation in the submandibular gland. Additionally, the levels of Th1/Th2/Th17 cytokines in the peripheral blood, submandibular gland tissues and saliva were all decreased. These results suggest that SHED begin to exert immunomodulatory effect in 14 weeks, while it is not sufficient for decreasing the inflammation in the submandibular gland in 14-week-old NOD mice. The protective effect on the secretory function and the immunomodulatory effect of SHED were more obvious in the later phase of the disease. In addition, SHED suppressed the peripheral inflammatory cytokines and regulated the local inflammatory microenvironment, which might be involved in the immunomodulatory effect of SHED.

The epithelial cells of salivary gland are the primary target of the immune attack. The impairment of epithelial cells directly results in the secretory dysfunction (Fox, 2012; Mitsias, Kapsogeorgou, & Moutsopoulos, 2006). Here, we found that apoptosis was increased in the submandibular glands of NOD mice, which is consistent with our previous study (Su et al., 2014). SHED treatment decreased apoptosis. In addition, another study has reported that the autophagy in conjunctival epithelial cells is upregulated in SS patients (Byun et al., 2017). However, whether there is autophagy in the diseased salivary gland is unknown. Here, we confirmed that autophagy was increased in the submandibular glands as the disease progresses. SHED treatment decreased autophagy. The decreased apoptosis and autophagy induced by SHED can alleviate the damage to the submandibular glands and protect their secretory function.

Next, we evaluated the safety of the SHED treatment and found that it had no effect on the histology of the parotid gland, heart, lung, liver, pancreas, spleen, kidney or intestine tissues. In addition, SHED have no effect on the structure or composition of the faecal microbiota. These above results suggest that SHED treatment is an effective and safe therapy for SS.

In the treatment of the disease, both BMSCs and ADSCs are capable of homing specifically to the injured regions after intravenous injection (Huang et al., 2010; Kale et al., 2003). Adipose-derived stem cells could alleviate the inflammatory response and improve the condition of the blood–brain barrier. The injected ADSCs were found in the brain ischaemic penumbra area (Chi et al., 2018). In the treatment of chemotherapy-induced ovarian damage, the infused BMSCs were detected in the ovarian interstitial tissues within 2–8 weeks after transplantation (Fu, He, Xie, & Liu, 2008). When using BMSCs to treat NOD mice, after the BMSCs infusion, the cells could migrate towards the inflammatory site (submandibular gland), as well as the kidney, lung, spleen and liver tissues of NOD mice from the first day. Next, the BMSCs distributed in the kidney, spleen and lung were decreased 1 week after infusion; however, a high number of BMSCs remained in the salivary gland. Further study indicated that the BMSCs located in the submandibular gland exert their immunoregulatory functions and, consequently, exhibit therapeutic effects (Xu et al., 2012). However, it is unknown whether SHED could move to the injured regions and then exert their function. In this study, we found that the infused SHED did not migrate to the submandibular gland. SHED distributed in the liver and spleen from the first hour and lasted for 7 days. The distribution of SHED in the liver might be affected by blood supply. Interestingly, the SHED in the spleen, the largest immune organ, attracted our attention. Further study is to confirm whether the spleen is involved in the SHED-induced protective effect on secretory function and to clarify the mechanism by which SHED exert immunoregulation.

MSCs exhibit immunomodulatory effects and the exact mechanism is due to the specific microenvironment that they encounter (Shi et al., 2012). Oral stem cells have been reported in terms of inhibition of inflammation and regeneration (Huang et al., 2009; Sloan & Smith, 2007). In the treatment of systemic lupus erythematosus, SHED show immunosuppression effects *via* recovering the Treg/Th17 ratio and decreasing the Th17 cell levels in the peripheral blood (Yamaza et al., 2010). In a study that used BMSCs to treat NOD mice, the infused BMSCs directed $CD4^+$ T cell responses in the spleen and submandibular lymph nodes towards Treg and Th2, while inhibiting Th17 and Tfh responses, and ultimately alleviated disease symptoms (Xu et al., 2012). However, it is unknown whether SHED could regulate $CD4^+$ T-cell differentiation and then inhibit inflammation. In our study, comparing with those in 7-week-old mice, the Th1 and Tfh proportions were increased in spleen of 14-week-old mice. SHED significantly decreased Th1

and Tfh cell and increased Treg cell. These results suggest that SHED direct T cells towards Treg cells, and suppressed Th1 and Tfh cells, which might be involved in the immunomodulatory effects of SHED.

In summary, our results demonstrate that SHED exert a protective effect on the secretory function of the salivary gland. SHED alleviate hyposalivation caused by SS *via* decreasing the peripheral inflammatory cytokines, regulating the local inflammatory microenvironment, and decreasing apoptosis and autophagy in the submandibular glands. The SHED regulated in T-cell differentiation might be involved in the immunomodulatory effects. This study will reveal a new effect of SHED and provide a therapeutic strategy for hyposalivation.

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CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTIONS

Z.-H. Du and C. Ding performed experiments, analysed the data and drafted the manuscript. Q. Zhang carried out faecal genomic DNA analysis. Y. Zhang and X. Y. Ge analysed the data and wrote the manuscript. S.-L. Li and G.-Y. Yu conceived and designed the study, and wrote the manuscript.

ORCID

Chong Ding https://orcid.org/0000-0002-1834-4421
Qian Zhang https://orcid.org/0000-0003-2448-6597

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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