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Original Article

Tripterygium glycosides and mesenchymal stem cells combination alleviated Sjögren's syndrome Features in mice and modulated the GRO-α/CXCR2 axis

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Abstract: Objective: To investigate the efficacy and potential mechanisms of tripterygium glycosides (TG) and human umbilical cord mesenchymal stem cells (hUC-MSCs) in the treatment of primary Sjögren's syndrome (PSS). Methods: Experimental Sjögren's syndrome (ESS) mice were administered TG (20 or 40 mg/kg/d, via oral gavage) and/or hUC-MSCs (1 × 10^6 cells/mouse, via tail vein injection) for a duration of 21 consecutive days. The parameters analyzed included saliva volume, lymphocytic infiltration in submandibular glands (SGs), serum cytokines, immunoglobulins, and the expression of the growth-related oncogene-alpha (GRO-α)/CXC chemokine receptor 2 (CXCR2) axis, as well as aquaporins (AQP1, AQP5). Results: Co-treatment with TG and hUC-MSCs resulted in a significant increase in saliva volume and a reduction in lymphocytic infiltration within the SGs. Additionally, this treatment decreased levels of pro-inflammatory cytokines (interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α) while enhancing the levels of anti-inflammatory cytokines (IL-10, IL-13) in serum, and modulated T helper (Th) 1 and Th2 cell populations. Furthermore, the combination therapy effectively suppressed elevated levels of immunoglobulins (immunoglobulin (Ig) M, IgG, IgA) in ESS mice. Mechanistically, the co-treatment inhibited the GRO-α/CXCR2 axis and upregulated the expression of AQP1 and AQP5 in SGs. Conclusion: The findings of this study suggest that co-treatment with TG and hUC-MSCs mitigates the symptoms of PSS by modulating inflammatory and immune responses and by inhibiting the GRO-α/CXCR2 axis.

Keywords: Sjögren's syndrome, traditional Chinese medicines, tripterygium glycosides, umbilical cord mesenchymal stem cells, GRO-α/CXCR2 axis

Introduction

Primary Sjögren's syndrome (PSS) is an autoimmune disorder characterized by the infiltration of mononuclear cells and the subsequent destruction of salivary and lacrimal glands, leading to the development of xerostomia and xerophthalmia [1]. Due to the complex and ambiguous pathogenesis of PSS, the range of currently effective therapies for this condition has been significantly constrained.

In traditional Chinese medicine, PSS is classified as a Bi syndrome characterized by dryness. The fundamental etiological factors and pathogenesis of PSS are attributed to deficiencies in Qi and Yin, as well as blood stasis [2]. Traditional Chinese medicines (TCMs) have been commonly used in China for treating PSS because of their remarkable curative effects, multitargeted actions, minimal side effects, and low cost [3]. Lycium barbarum ameliorated salivary gland inflammation and hypofunction in PSS mice, which was associated with modulation of T cell differentiation [4]. Catalpol, a bioactive constituent extracted from Rehmanniae Radix, has been shown to modulate the interaction between T and B lymphocytes, thereby inhibiting the progression of PSS [5]. The utilization of a combinatorial approach involving GanLu-Yin, Sang-Ju-Yin, and Xuefu-Zhuyu-Decoction has been extensively studied to enhance salivary flow rates in patients diagnosed with Sjögren's syndrome [6]. Tripterygium glycosides (TG), derived from the dry root of the traditional Chinese herb Tripterygium wilfordii, is a non-

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steroidal immunosuppressant with therapeutic potential. It is known to replenish qi, nourish Yin, clear heat, and promote the production of body fluids. TG has shown efficacy in treating various diseases, including rheumatoid arthritis [7], nephropathy [8], skin disease [9], and liver injury [10]. Interestingly, a recent review indicated that TG may be effective in treating PSS in clinical settings by increasing saliva flow rate and enhancing lacrimal gland function [11]. However, the molecular mechanisms by which TG inhibits the progression of PSS have not yet been investigated.

Considering the complex pathogenesis of PSS, recent studies have identified the GRO-α/CXCR2 axis as a promising target. This axis is not only upregulated in the salivary glands of PSS patients but also plays a role in regulating key components such as ADAM17, and it is associated with aquaporin disruption [12, 13]. We hypothesized that the therapeutic effects of TG and hUC-MSCs may be mediated, at least in part, by modulating a specific axis that connects immune cell infiltration to glandular functional impairment.

Mesenchymal stem cells (MSCs) are increasingly recognized as the cornerstone of stem cell therapy due to their unique ability for renewal and differentiation. The clinical benefits of mesenchymal stem cells derived from the human umbilical cord (hUC-MSCs) are well established, owing to their low immunogenicity, rich content, and non-invasive collection methods. Previous studies have indicated that MSCs possess significant therapeutic potential in the treatment of PSS [14, 15]. Previous research has indicated that the administration of UCMSC-derived exosomes (UCMSC-Exos) leads to a reduction in the elevated autophagy levels observed in CD4+ T cells of patients diagnosed with PSS [15]. Additionally, UCMSC-Exos demonstrated regulatory effects on CD4+ T cell proliferation and early apoptosis, inhibited the differentiation of Th17 cells, promoted the differentiation of Treg cells, and restored the equilibrium between Th17 and Treg cells in individuals with PSS [15]. Moreover, MSCs in mice effectively inhibited the reduction in salivary flow rate and lymphocyte infiltration within the salivary glands of NOD mice [16].

This study examines the effects of TG, UC-MSCs, and their combination on PSS, with a particular focus on inflammation in the submandibular gland, anti-inflammatory effects, and Th cell regulation. Additionally, we explore the mechanisms underlying these therapies using an experimental mouse model of Sjögren's syndrome (SS).

Materials and methods

Drug

Tripterygium glycosides (TG) tablets were purchased from Zhejiang Deende Pharmaceutical Co., Ltd. (Country Medicine Accurate Character Number: Z33020422, Shaoxing, Zhejiang, China). Stock solutions of TG (10 mg/ml) were prepared with distilled water.

Culture of MSCs

The UC-MSCs were purchased from Medipost Co., Ltd. (Korea). The UC-MSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing low glucose, supplemented with 10% fetal bovine serum (FBS, 10099, Gibco, Paisley, UK), and maintained at a temperature of 37°C in an atmosphere of 5% CO_2.

Animals

Ninety-five female C57BL/6 mice (8-week-old) were procured from Chengdu Dossy Experimental Animals CO., LTD. (Chengdu, Sichuan; SCXY (Chuan) 2020-034). The mice were maintained according to ARRIVE guidelines. The procedures were approved by the Animal Research Ethics Committee of the Hebei Province Hospital of Chinese Medicine (DWLL202203079). The mice were raised in a standard environment with 12-h day/night cycles, 26 ± 2°C temperature, and 50 ± 5% relative humidity. All animals were granted unrestricted access to food and water.

ESS modeling

The ESS mouse model was established as previously described [17, 18]. In summary, the submandibular gland (SG) of the mouse was isolated and subsequently homogenized in ice-cold phosphate-buffered saline (PBS). Following homogenization, centrifugation was conducted at 12,000 rpm for 20 min at a temperature of 4°C. The protein concentration in the resulting supernatant was determined using a BCA™ protein assay kit (23225, Thermo Scientific,

Rockford, IL, USA). The supernatant was then emulsified in Freund's complete adjuvant at a concentration of 4 mg of protein per ml; 344289, Sigma-Aldrich, St. Louis, MO, USA) and administered subcutaneously to C57BL/6 mice at a volume of 0.1 ml to induce ESS on days 0 and 7. Two weeks later, a booster immunization was administered using an equal volume of Freund's incomplete adjuvant. Mice in the control group received injections of adjuvant only.

Animal grouping and treatment

In week 6, the failed mice models were culled based on saliva flow. In Experiment 1, C57BL/6 mice were randomly assigned to four groups (n=8): the control group, the ESS model group, the low-dose Tripterygium glycosides group (TG-low), and the high-dose Tripterygium glycosides group (TG-high). In Experiment 2, the C57BL/6 mice were randomly allocated into six groups (n=8): the control group, the ESS model group, the TG low dose group (TG-low), the TG high dose group (TG-high), the UC-MSCs group, and the TG combined with UC-MSCs group (TG + UC-MSCs). For the TG-low and TG-high groups, the ESS mice received oral administration of 20 mg/kg/d and 40 mg/kg/d of TG for 21 consecutive days [19, 20], respectively. For the UC-MSCs group, the MSCs were suspended in PBS at passage 4 and administered to mice via the tail vein at a concentration of 1×10^6 cells [21, 22] per mouse for a duration of 21 consecutive days. For the TG + UC-MSCs group, the ESS mice received a daily gavage of 40 mg/kg/d TG and were administered UC-MSCs (1 × 10^6 cells/mouse/week) via the tail vein for 21 days. The control and model groups were given an equivalent volume of distilled water/PBS through either oral gavage or tail vein injection. At the end of the experiment, the mice were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital, followed by euthanasia through CO_2 inhalation. Samples from the submandibular gland (SG) and blood were collected and stored at -80°C for later analyses.

Salivary flow measurements

Salivary flow in the mice was measured at 6, 7, 8, and 9 weeks post-modeling. After anesthetization, weighed sterile cotton gauze was placed under the mice's tongues for 10 min. The change in weight of the sterile cotton gauze indicated the amount of salivary flow.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6 (H007-1-1), IL-1β (H002-1-2), tumor necrosis factor (TNF)-α (H052-1-2), IL-10 (H009-1-2), IL-13 (H011), immunoglobulin M (IgM; H109-1-1), immunoglobulin G (IgG; H106-1-1), and immunoglobulin A (IgA; H108-1-2) in mouse serum were quantified using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm using an enzyme-linked immune monitor (Multiskan FC, Thermo Fisher Scientific, Inc., Waltham, USA).

Hematoxylin and eosin (H&E) stain

To prepare the submandibular gland (SG) of mice for histological examination, the tissue underwent xylene and ethanol treatment for deparaffinization and rehydration before hematoxylin staining. This was followed by dehydration in graded alcohols, clearing with xylene, and eosin counterstaining prior to mounting with resin. Histopathological changes in the SG were observed using a light microscope (Olympus BH2, Tokyo, Japan).

Immunohistochemistry (IHC) stain

Protein levels of growth-related oncogene-alpha (GRO-a) and CXC chemokine receptor 2 (CXCR2) in SG were assessed via IHC staining. The SG were dewaxed and hydrated, followed by antigen retrieval and inhibition of endogenous catalase. Non-specific antigen blocking was achieved using goat serum, and the blocking solution was subsequently removed. The sections were then incubated overnight with either rabbit polyclonal GRO-α antibody (ab269939, Abcam, MA, USA, 1:100) or rabbit monoclonal CXCR2 antibody (ab129991, Abcam, MA, USA, 1:2000). After rinsing with PBS buffer containing 1% BSA, sections were incubated at 37°C with goat anti-rabbit IgG (SP9001, ZsBio, China) for 30 min. IHC images were captured using a digital trinocular camera microscope system (BA400Digital, Motic Instruments, Inc., Baltimore, MD, USA), and positive expressions were analyzed with Image-Pro Plus 6.0.

Immunofluorescence (IF) stain

Paraffin sections of the SG were embedded and sectioned to a thickness of 8 μm. The sections were air-dried on glass slides, permeabi-

lized with 10% TritonX-100 to create tissue holes, and blocked with 5% BSA while incubating in a light-protected box. Subsequently, the sections were incubated with Th1 (CD4+/IFN-γ+) antibodies (CD4, ab133616, Abcam, MA, USA, 1:500; IFN-γ, bs-0481R, Bioss, Beijing, China, 1:100) or Th2 (CD4+/IL-4+) antibodies (IL-4, 500-P54, Peprotech, Rocky Hill, NJ, USA, 1:200). The slides were then incubated overnight in a wet box at 4°C in darkness. The following day, a FITC goat anti-rabbit IgG was prepared at a dilution ratio of 1:100, applied to the washed tissue sections, and incubated in darkness. After washing with PBS, the slides were treated with 4',6-Diamidino-2-Phenylindole (D9542, DAPI, Sigma-Aldrich, St. Louis, MO, USA) and blocked with an anti-fluorescence quenching agent, then covered with coverslips. Imaging was conducted under a fluorescence microscope (Olympus IX81-ZDC, Tokyo, Japan) at a magnification of 400×.

Western blot analysis

Protein quantification was performed using the BCA kit (Sigma-Aldrich, St. Louis, MO, USA) Based on the quantitative results, a loading volume of 30 μg per well was utilized for sampling, and total protein was separated via 10% SDS-PAGE electrophoresis. Following this, the membrane was transferred to a PVDF membrane, blocked with 5% skim milk, and incubated with the appropriate primary antibodies at 4°C overnight. The antibodies used were: GRO-α (ab206411, Abcam, MA, USA, 1:100), CXCR2 (A3301, Abclonal, Wuhan, Hubei, China, 1:500), AQP5 (ab305303, Abcam, MA, USA, 1:1000), AQP1 (A4195, Abclonal, Wuhan, Hubei, China, 1:100), and β-actin (BM0627, Boster, Wuhan, Hubei, China, 1:1000). Following washing with Tris-buffered saline/0.1% Tween (TBST), the appropriate amount of HRP Goat anti-Rabbit IgG (ab6721, Abcam, MA, USA, 1:2,000) was incubated at room temperature for 1.5 h. Finally, the ECL color development solution was utilized for chromogenic detection in conjunction with the ECL system (K003, Affinity Biosciences, Cincinnati, OH, USA).

Statistical analysis

All data are shown as the mean ± standard deviation. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test for multiple comparisons. Statistical analyses were performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). A *P*-value <0.05 was considered statistically significant.

Results

TG dose-dependently improved salivary gland function in the Experimental Sjögren's syndrome (ESS) model

After 6 weeks of modeling, the ESS model group exhibited a significantly reduced salivary flow rate compared to the normal group (**Figure 1A**). In the ESS mice treated with a low dose of TG, there were no significant changes in salivary flow after 1 (P>0.05) and 2 (P>0.05) weeks. However, by the third week, salivary flow significantly increased (P<0.05, **Figure 1A**). In contrast, salivary secretion in ESS mice treated with high doses of TG significantly increased after 1 (P<0.05), 2 (P<0.01), and 3 (P<0.01) weeks (**Figure 1A**).

TG dose-dependently decreased inflammatory response in the ESS model

In ESS model mice, serum levels of IL-6 (P<0.01, **Figure 1B**), IL-1β (P<0.01, **Figure 1C**), and TNF-α (P<0.01, **Figure 1D**) were elevated, while levels of IL-10 (P<0.01, **Figure 1E**) and IL-13 (P<0.01, **Figure 1F**) were decreased. In the TG low-dose group, IL-1β levels were significantly reduced (P<0.05, **Figure 1E**), but other immunomodulatory factors showed no significant changes (P>0.05, **Figure 1B**, **1D**, **1F**). Conversely, the high-dose TG treatment in ESS mice resulted in a significant reduction in serum IL-6 (P<0.01, **Figure 1B**), IL-1β (P<0.01, **Figure 1C**), and TNF-α (P>0.01, **Figure 1D**), along with substantial increases in IL-10 (P<0.01, **Figure 1E**) and IL-13 (P<0.01, **Figure 1F**) levels.

TG dose-dependently attenuated the pathological changes of SG

The submandibular glands of ESS mice displayed more severe lymphocyte infiltration compared to the normal group, leading to the formation of lymphocyte foci (**Figure 1G**). Additionally, the lobular structure of the salivary glands was disrupted, and local small blood vessels exhibited dilation and congestion due to inflammation (**Figure 1G**). Notably, the group treated with TG at a concentration of 40 mg/kg showed significant improvement in lymphocyte

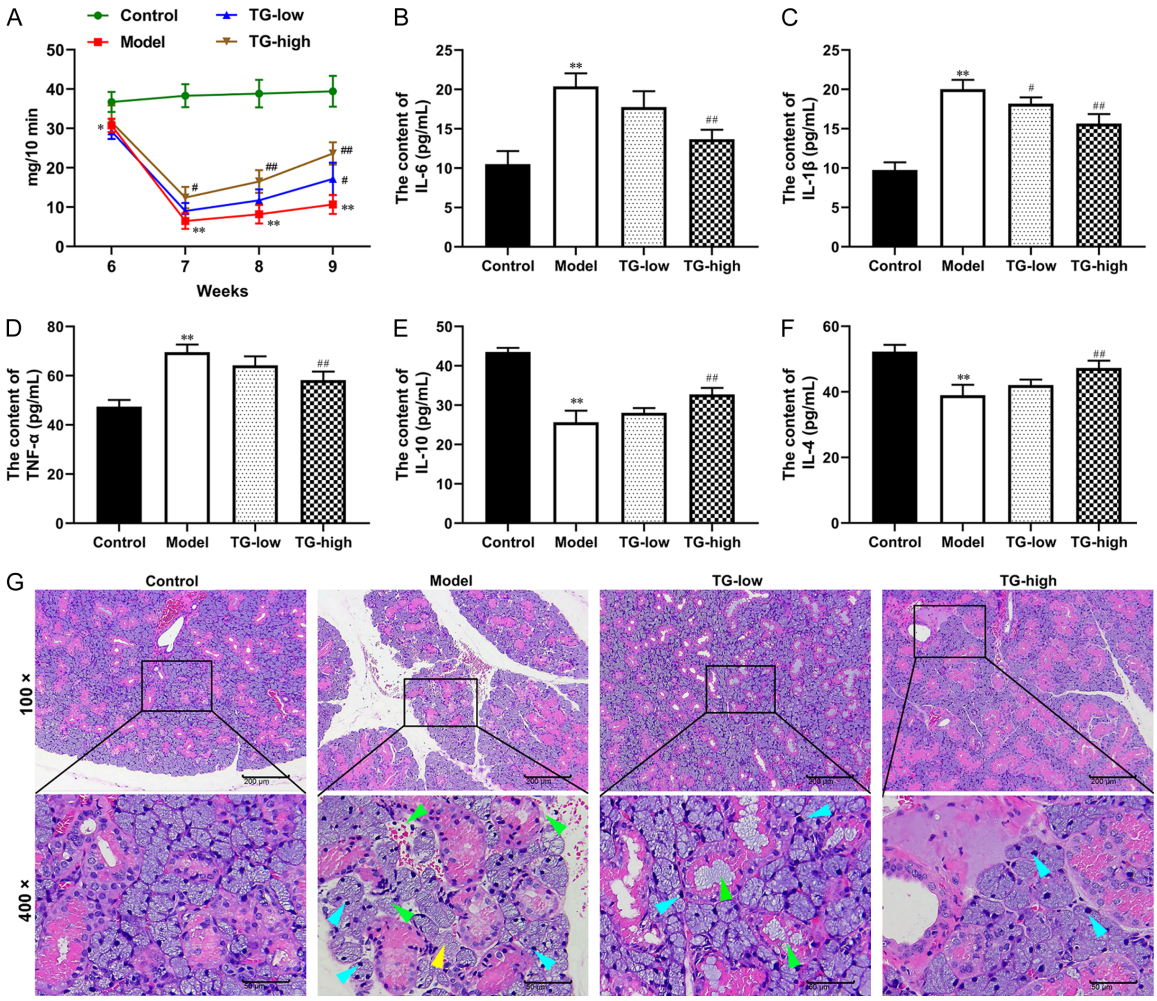

Figure 1. TG dose-dependently improved salivary gland function and decreased inflammatory response in the ESS model. (A) Salivary secretion was examined within the 6th to the 9th week following ESS modeling. The concentration levels of IL-6 (B), IL-1β (C), TNF-α (D), IL-10 (E), and IL-13 (F) in the serum of mice were quantified through the enzyme-linked immunosorbent assay (ELISA). (G) The submandibular gland was subjected to Hematoxylin and eosin (H&E) staining, with magnifications of ×100 and ×400. Lymphocytes are represented by blue arrows, acinar necrosis by yellow arrows, and ruptured/dilated granular convoluted tubules by green arrows. $^{**}P<0.01$ vs. Control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Model.

infiltration within the submandibular gland compared to the ESS model group (**Figure 1G**).

TG combined with UC-MSCs ameliorated salivary gland function in the ESS model

As depicted in **Figure 2A**, a significant reduction in salivary flow was observed in the model group starting from 6 weeks post-modeling, compared to the control group (P<0.05). At week 9, both the TG low dose (P<0.05) and UC-MSCs (P>0.05) alone weakly enhanced the saliva amount of ESS mice (**Figure 2A**). After a 2-week administration period, both the TG high dose group and the TG high dose combined with the UC-MSCs group exhibited a significant increase in saliva flow compared to the model group (P<0.01, **Figure 2A**). Meanwhile, the combination treatment outperformed TG alone (P<0.05, **Figure 2A**), indicating a protective effect on saliva secretion function in ESS mice.

TG combined with UC-MSCs inhibited serum inflammatory cytokines in ESS mice

SS is a chronic autoimmune disease characterized by a large presence of inflammatory cells and cytokines. We investigated the effects of TG and UC-MSCs on inflammation associated with SS. ELISA results indicated that both TG

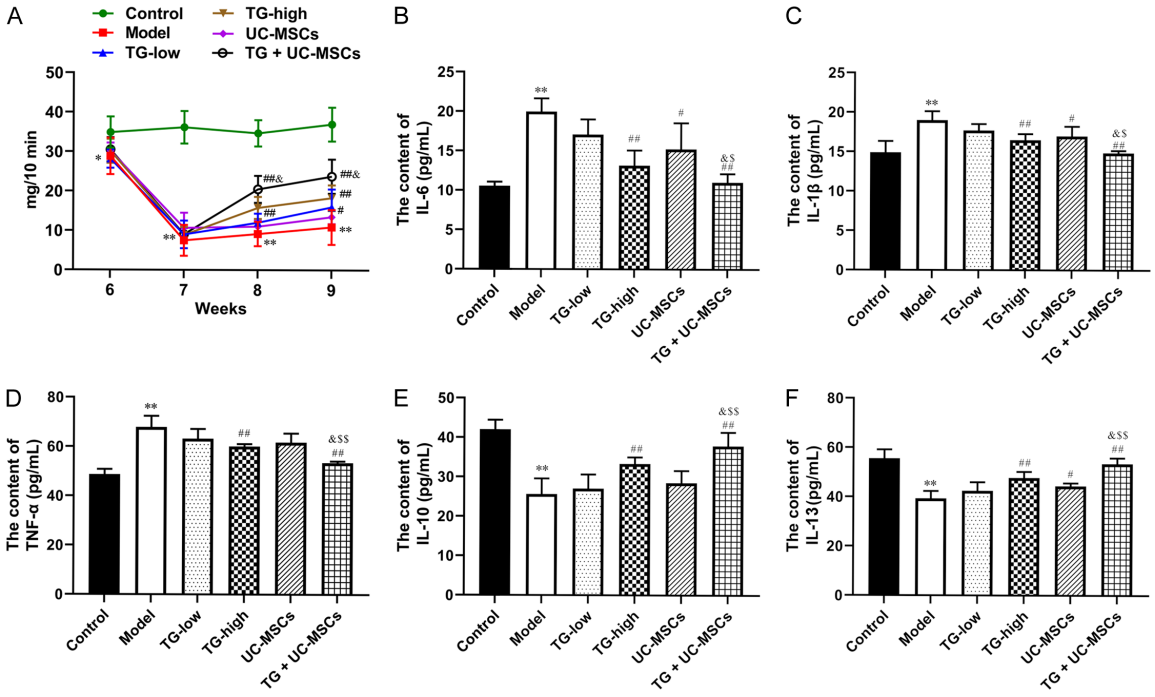

Figure 2. TG combined with UC-MSCs ameliorated salivary gland function and inhibited serum inflammatory cytokines of ESS mice. (A) Saliva secretion was monitored from the 6th week to the 9th week after ESS modeling. The contents of IL-6 (B), IL-1β (C), TNF-α (D), IL-10 (E), and IL-13 (F) in mouse serum were quantified using ELISA. $^{**}P<0.01$ vs. Control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Model, $^{\&}P<0.05$ vs. TG-high, $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs. UC-MSCs. Data are shown as the mean ± SD. n=8.

low dose and UC-MSCs alone resulted in minor decreases in pro-inflammatory factors IL-6 (P>0.05 or P<0.05, **Figure 2B**), IL-1β (P>0.05 or P<0.05, **Figure 2C**), and TNF-α (P>0.05, **Figure 2D**), along with slight increases in anti-inflammatory factors IL-10 (P>0.05, **Figure 2E**) and IL-13 (P>0.05 or P<0.05, **Figure 2F**) in serum. In addition, the high-dose TG treatment and the combination of TG high dose with UC-MSCs resulted in significant decreases in the expression levels of pro-inflammatory factors (P<0.01, **Figure 2B-D**) and down-regulated levels of anti-inflammatory factors (P<0.01, **Figure 2E**, **2F**) in the serum of ESS mice.

TG combined with UC-MSCs suppressed lymphocytic infiltration in SG of ESS mice

Histological examination of the SG at week 9 revealed significant differences between the model group and the control group. The model group exhibited massive infiltration of lymphocytes, necrotic acini, and rupture of granular convoluted tubules (**Figure 3**). While the TG low dose and UC-MSCs group showed some improvement in SG lesions in ESS mice, the degree of improvement was modest (**Figure 3**). In contrast, the TG high dose and the combination of TG with UC-MSCs significantly reduced both lesions and lymphocytic infiltration (**Figure 3**). Notably, the improvement was markedly greater in the combined therapy group compared to the TG high dose alone (**Figure 3**).

TG and UC-MSCs treatment decreased immunoglobulin levels in the serum of ESS mice

We subsequently assessed the serum immunoglobulin levels in ESS mice using ELISA. The model group exhibited significant elevations in serum immunoglobulin levels of IgM (P<0.01, **Figure 4A**), IgG (P<0.01, **Figure 4B**), and IgA (P<0.01, **Figure 4C**) compared to the control group. The TG low dose resulted in a decrease in IgG levels in the serum of ESS mice (P<0.05, **Figure 4B**). Furthermore, treatments with TG high dose, UC-MSCs, and the combination of TG high dose with UC-MSCs led to the elimination of IgM (P<0.05 or P<0.01, **Figure 4A**), IgG (P<0.05 or P<0.01, **Figure 4B**), and IgA (P<0.05

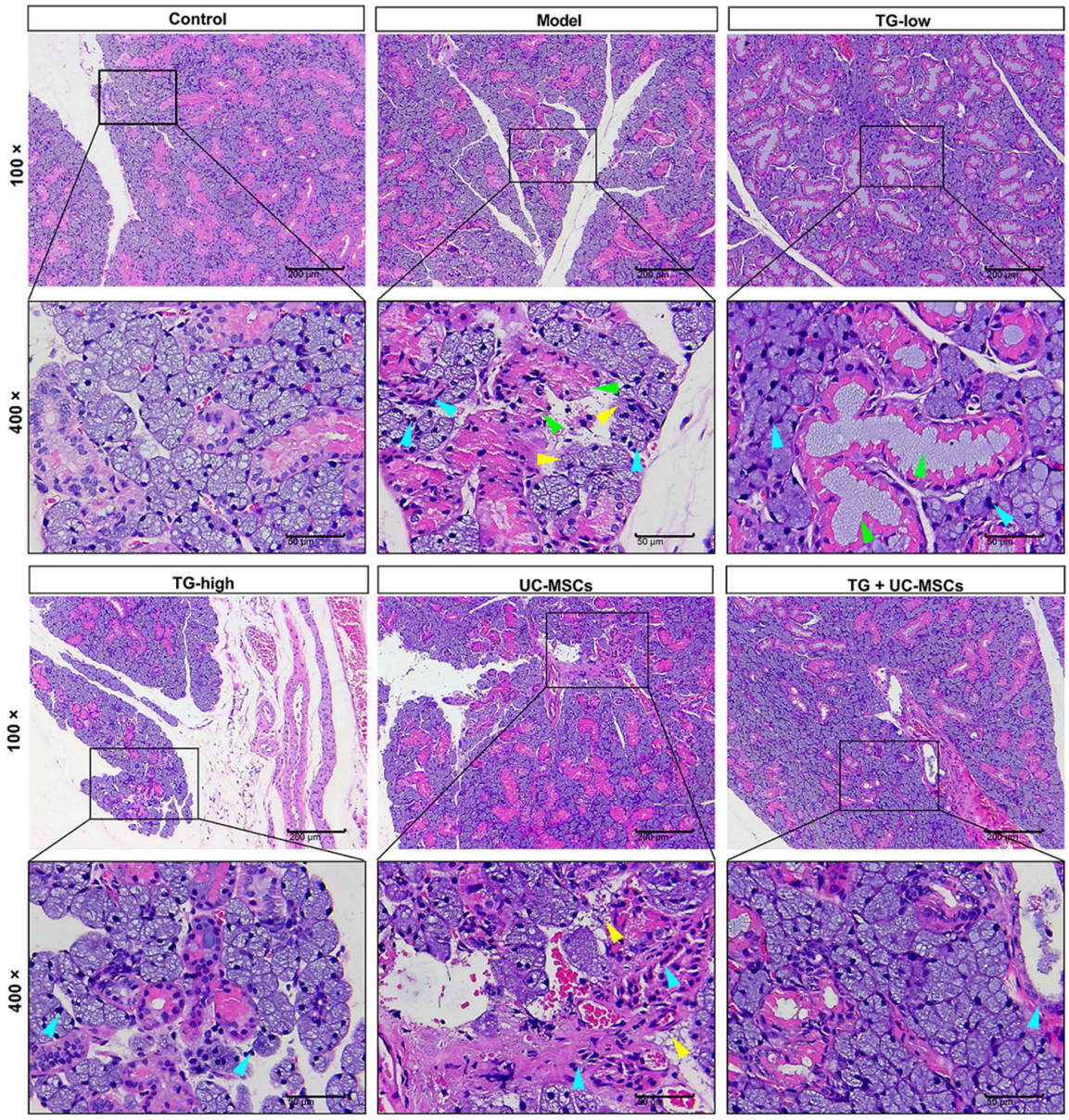

Figure 3. TG combined with UC-MSCs suppressed lymphocytic infiltration in SG of ESS mice. H&E stain of the submandibular gland. The magnification is ×100 and ×400. Blue arrows (lymphocytes), yellow arrows (acinar necrosis), and green arrows (ruptured/dilated granular convoluted tubules).

or P<0.01, **Figure 4C**) levels. Among these groups, the combined treatment demonstrated the strongest effects (**Figure 4A-C**).

TG and UC-MSCs treatment reduced the levels of Th1 and Th2 cells in SG of ESS mice

It has been suggested that Th1 and Th2 play important roles in the pathogenesis of SS [23]. We investigated the levels of IFN-γ+CD4+ T cells (Th1) and IL-4+CD4+ T cells (Th2) in the SG of ESS mice using immunofluorescence staining. Our results indicated that TG low/high dose, as well as UC-MSCs treatment alone, significantly decreased the levels of Th1 (P<0.01, **Figure 5A**) and Th2 (P<0.05 or P<0.01, **Figure 5B**) cells in the SG of ESS mice. Interestingly, the percentage of Th1 (P<0.05 or P<0.01) and Th2 (P<0.05 or P<0.01) cells in the TG high dose combined with UC-MSCs group was significantly lower than in the TG or UC-MSCs treatment groups alone (**Figure 5A** and **5B**). These findings support the idea that TG and UC-MSCs treatment alleviates the Th1 and Th2 immune imbalance in the SG of ESS mice.

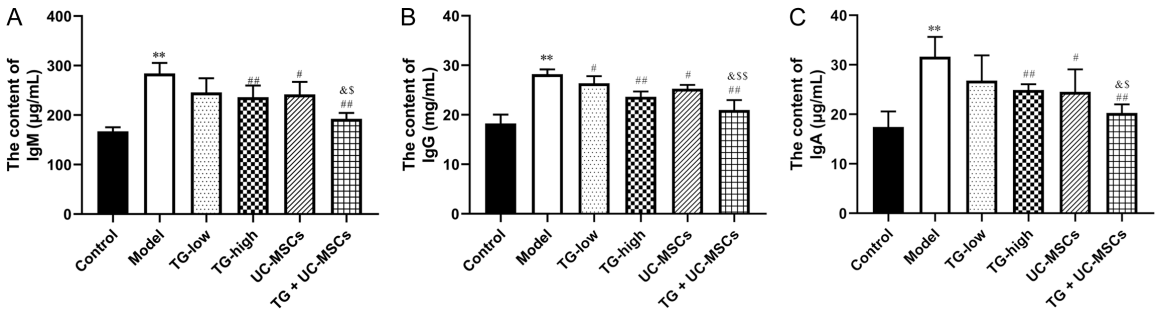

Figure 4. TG and UC-MSCs treatment decreased immunoglobulin levels in serum of ESS mice. The content of IgM (A), IgG (B), and IgA (C) in mouse serum was quantified using by ELISA. $^{**}P<0.01$ vs. Control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Model, $^{\&}P<0.05$ vs. TG-high, $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs. UC-MSCs. Data are presented as the mean ± SD. n=8.

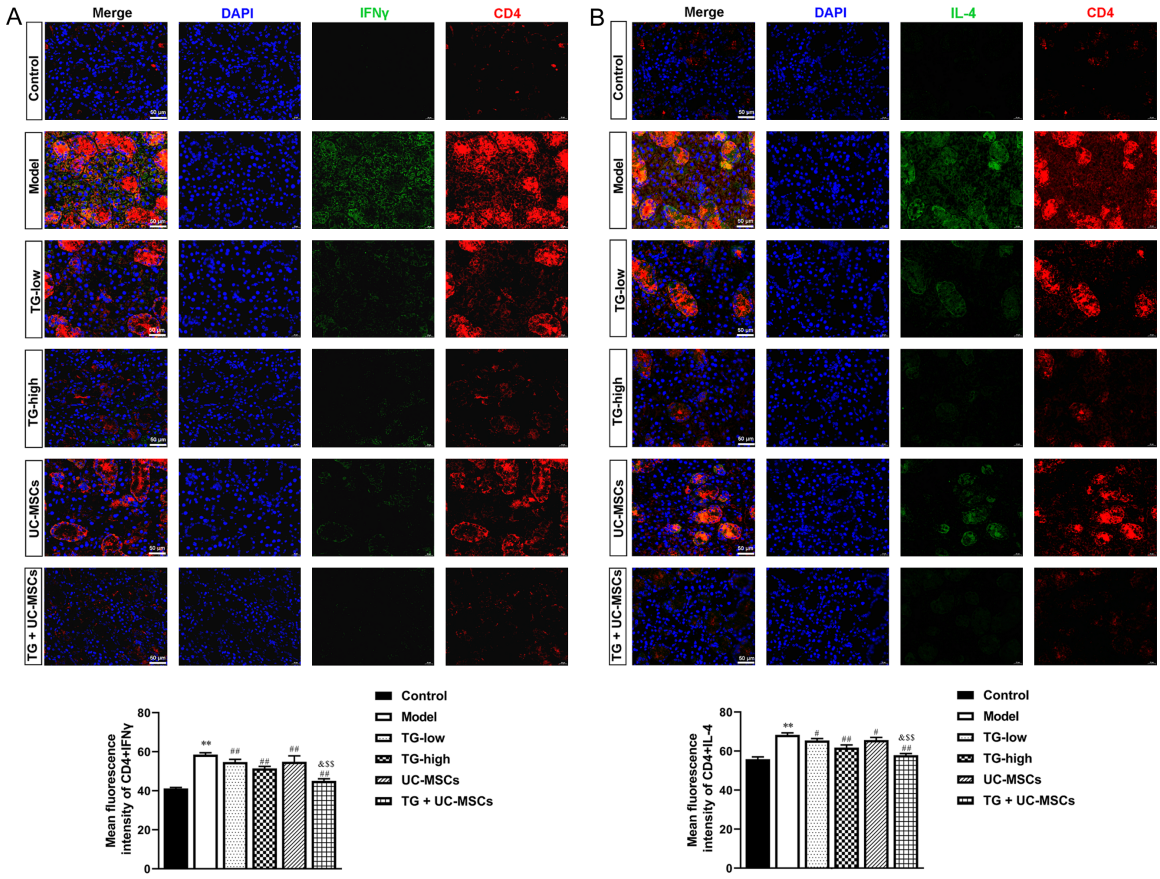

Figure 5. TG and UC-MSCs treatment reduced the levels of Th1 and Th2 cells in SG of ESS mice. A. Immunofluorescence (IF) staining for IFNγ and CD4 expression in the submandibular gland of ESS mice. B. Representative immunofluorescence staining for CD4 and IL-4. The magnification is ×400. $^{**}P<0.01$ vs. Control, $^{\#\#}P<0.01$ vs. Model, $^{\&}P<0.05$ vs. TG-high, $^{\$\$}P<0.01$ vs. UC-MSCs. Data are indicated as the mean ± SD. n=3.

TG and UC-MSCs treatment reduced the activation of the GRO-α/CXCR2 axis in SG of ESS mice

To further investigate the critical roles of the GRO-α/CXCR2 complex during ESS progression, we examined whether TG or UC-MSCs treatment affects the GRO-α/CXCR2 axis in the ESS group. IHC and Western blot analyses revealed that both low and high doses of TG, along with UC-MSCs treatment alone, reduced the expression of GRO-α ($P<0.05$ or $P<0.01$)

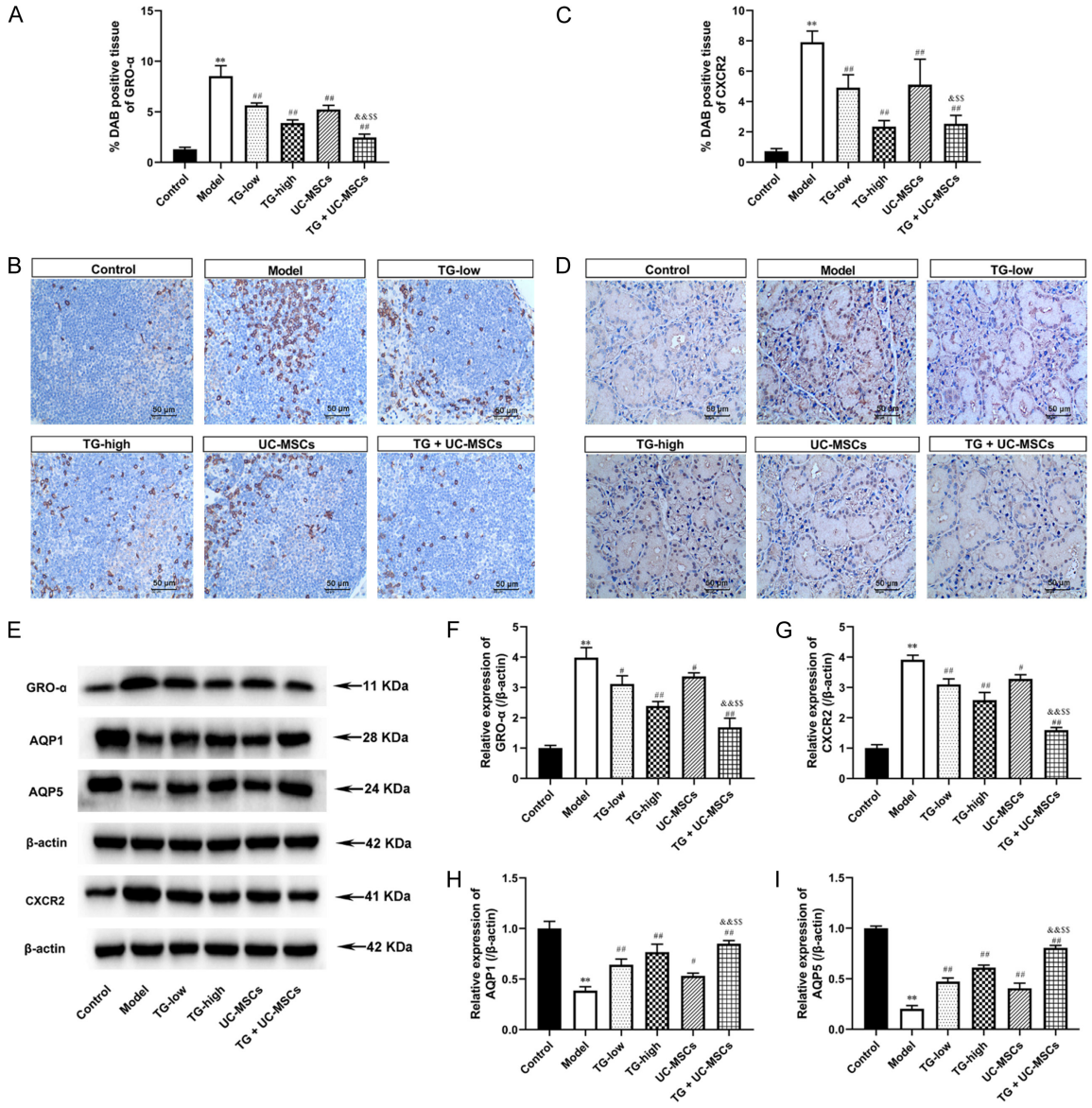

Figure 6. TG and UC-MSCs treatment reduced the activation of the GRO-α/CXCR2 axis in SG of ESS mice. Immunohistochemistry for GRO-α (A and B) and CXCR2 (C and D). (E-I) Western blot analysis for the expression of GRO-α (E and F), CXCR2 (E and G), AQP1 (E and H), and AQP5 (E and I) in the submandibular gland of ESS mice. β-actin served as the loading control. $^{**}P<0.01$ vs. Control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Model, $^{\&\&}P<0.05$ vs. TG-high, $^{\$\$}P<0.01$ vs. UC-MSCs. Data are presented as the mean ± SD. n=3.

and CXCR2 (P<0.05 or P<0.01) in the SG of ESS mice (**Figure 6A-G**). The combination of TG high dose and UC-MSCs demonstrated a more pronounced effect on the activation of the GRO-α/CXCR2 signaling pathway compared to either treatment alone (P<0.01, **Figure 6A-G**). Moreover, we observed decreased expression of AQP1 (P<0.05 or P<0.01) and AQP5 (P<0.05 or P<0.01) in the SG of ESS mice following stimulation with TG low/high doses and UC-MSCs treatment (**Figure 6E**, **6H**, **6I**). Co-treatment with TG high dose and UC-MSCs significantly enhanced the expression of AQP1 and AQP5 compared to either treatment alone (P<0.01, **Figure 6E**, **6H**, **6I**). These results suggest that the GRO-α/CXCR2 axis is involved in the protective effects of TG and UC-MSCs on ESS, potentially linked to increased expression of AQP1 and AQP5.

Discussion

In vivo, our study demonstrated that the application of TG and UC-MSCs significantly im-

proved ESS symptoms, increased saliva volume, and mitigated abnormal changes in the submandibular gland pathology of ESS mice. Treatment with TG and UC-MSCs treatment reduced inflammatory cytokines and immunoglobulin levels in the peripheral blood of these mice. In addition, this treatment diminished both the Th-1 and Th-2 phenotypes, as well as the activity of the GRO-α/CXCR2 axis in the submandibular gland. These findings suggest that the combination therapy alleviates PSS by inhibiting the GRO-α/CXCR2 axis, a pathway chosen for investigation due to its documented upregulation in PSS salivary glands and its established role in mediating leukocyte infiltration and gland dysfunction [12, 13]. While other mechanisms, such as the BAFF and IFN pathways, are undoubtedly significant in PSS, our study identifies the GRO-α/CXCR2 axis as a key mechanistic target for the co-treatment of TG and hUC-MSCs. Future research exploring the interactions between the GRO-α/CXCR2 axis and these other pathways would be highly valuable.

CD4 T cells perform their functions in four subpopulations: Th-1, Th-2, Th-17, and T follicular helper (Tfh) cell subsets [24] Th1 cells secreted IL-2, IFN-γ, and TNF-α to mediate immune-inflammatory responses [24]. Th2 cells secreted IL-4, IL-6, and IL-10 which were mainly responsible for the humoral immune response [24]. The balance between Th1 and Th2 cells is crucial for maintaining the homeostasis of the body's immune microenvironment. An imbalance in these cells can lead to either systemic or local abnormal immune responses. Previous studies have shown that damage to the exocrine glands in patients with PSS is closely linked to the degree of infiltration of Th1 and Th2 cells [25, 26]. The studies of NOD/LtJ IL-4 or IFN-γR knockout mice elucidated the important roles of Th1 and Th2 cells in the pathogenesis of PSS [27, 28].

Previous reports indicated that multiple TCM formulas and herbs are utilized to regulate Th1 and Th2 cytokine responses. Notably, Shenqi effectively balanced the Th1/Th2 ratio (IFN-γ/IL-4), demonstrating a significant anti-allergic effect in allergic rhinitis [29]. Wang-Bi tablets (WBTs), a Chinese patented medicine, relieved collagen-induced arthritis by maintaining the balance of Th1/Th2 cells [30]. In the present study, we found that treatment with TG and UC-MSCs reduced the levels of Th1 and Th2 cells in the submandibular gland tissue, leading to a significant improvement in the inflammatory infiltration of this gland in ESS mice. Additionally, TG and UC-MSCs treatment resulted in a marked decrease in peripheral serum levels of IL-6, IL-1β, and TNF-α, while simultaneously increasing the anti-inflammatory factors IL-10 and IL-13 in ESS mice. In addition, the combination therapy proved to be more effective than either treatment alone.

Recently, the discovery of aquaporins (AQPs), a group of membrane channel proteins, has shed light on the molecular mechanisms that regulate water permeability across various tissues [31]. AQPs provide a rapid and selective molecular pathway for water reabsorption through cellular membranes. Among the ten identified aquaporins, AQP1 and AQP5 have been confirmed to be present in human salivary gland tissues and are closely associated with saliva secretion. Evidence suggests that AQP1 and AQP5 significantly contribute to the pathological progression of certain salivary gland diseases, including PSS. Our findings indicate that the expression of AQP1 and AQP5 is decreased in the submandibular gland of ESS mice compared to the control group, but this decrease is reversed by treatment with TG (both low and high doses) and UC-MSCs.

Growth-regulated oncogene-alpha (GRO-α), also known as CXCL1, is a member of the CXC chemokine family and exerts its signaling effects through interaction with its receptor, CXCR2. The GRO-α/CXCR2 axis is implicated in a wide range of physiological and pathological processes, including normal functions such as leukocyte migration and homing, as well as pathological conditions like inflammation, infections, and malignant tumors. The GRO-α/CXCR2 receptor complex has been reported to play a role in angiogenesis and autoimmune diseases, including PSS, during inflammation [12, 32]. GRO-a and CXCR2 were highly expressed in salivary glands of PSS patients [12]. Meanwhile, pro-inflammatory cytokines induced the expression of the GRO-α/CXCR2 axis in human salivary gland epithelial cells [12]. Moreover, CXCR2 expression was increased in epithelial cells from SS salivary glands. This increase could be reduced by the ADAM17 inhibitor TAPI-1 and further blocked by GRO-α [13]. A recent review showed that the GRO-α/

CXCR2 system modulated PSS pathogenesis by suppressing ADAM17 activation [33]. In this study, the data indicated that the combination of TG and UC-MSCs significantly inhibited the activation of the GRO-α/CXCR2 axis in the submandibular gland of ESS mice. Our results suggest that TG and UC-MSCs play a role in regulating the progression of PSS through the GRO-α/CXCR2 axis. While our findings strongly support the inhibition of this axis as a key contributing factor, a limitation of this study is its exclusive focus on this predefined pathway for mechanistic investigation. Therefore, future research utilizing transcriptome sequencing approaches will be crucial for elucidating the broader gene regulatory networks and signaling pathways affected by the combined treatment of TG and hUC-MSCs.

In conclusion, our findings supported the application of TG and UC-MSCs for treating PSS. The combination of TG and UC-MSCs appears to exert immunoregulatory effects by reducing the levels of Th1 and Th2 cells, as well as the expression of the GRO-α/CXCR2 complex in the submandibular gland of ESS mice. Thus, co-treatment with TG and UC-MSCs may serve as a potent therapeutic strategy for PSS.

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Disclosure of conflict of interest

None.

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