Umbilical cord mesenchymal stem cells inhibit the differentiation of circulating T follicular helper cells in patients with primary Sjögren’s syndrome through the secretion of indoleamine 2,3-dioxygenase

Rui Liu¹, Dinglei Su¹, Min Zhou¹, Xuebing Feng¹, Xia Li² and Lingyun Sun¹

Abstract

Objective. The aim of this study was to investigate the effect of umbilical cord mesenchymal stem cells (UC-MSCs) on circulating T follicular helper (cTfh) cells in primary SS (pSS) patients.

Methods. The percentage of CXCR5⁺PD-1⁺CD4⁺ T cells in peripheral blood mononuclear cells (PBMCs) was analysed by flow cytometry. PBMCs were co-cultured with UC-MSCs by cell-to-cell contact or in a trans-well system. Naïve CD4⁺ T cells were isolated from PBMCs and then co-cultured with UC-MSCs under Tfh cell-polarizing conditions. The percentage of CXCR5⁺PD-1⁺CD4⁺ T cells, carboxyfluorescein succinimidyl ester (CFSE) fluorescence intensity and annexin V were determined by flow cytometric analysis. Real-time PCR and Luminex cytokine assay were performed to detect mRNA expression and supernatant protein levels. The activity of indoleamine 2,3-dioxygenase (IDO) was measured by HPLC.

Results. Increased frequency of cTfh cells was found in pSS patients and was positively correlated with serum anti-La/SSB levels and the European League Against Rheumatism SS Disease Activity Index score. In vitro, UC-MSCs suppressed the differentiation and proliferation of cTfh cells. Real-time PCR analysis showed significantly higher IDO mRNA expression on UC-MSCs when co-cultured with naïve CD4⁺ T cells under Tfh cell-polarizing conditions in pSS patients. However, IDO mRNA expression on UC-MSCs was only a little higher when UC-MSCs were co-cultured with naïve CD4⁺ T cells in a trans-well system. In addition, HPLC showed increased IDO enzymic activity in the supernatant of UC-MSCs co-cultured with naïve CD4⁺ T cells under Tfh cell-polarizing conditions in pSS. The addition of the IDO inhibitor 1-MT partly reversed the suppressive effect of UC-MSCs on the differentiation of cTfh cells.

Conclusion. These results suggest an inhibitory effect of UC-MSCs on the differentiation of cTfh cells via the secretion of IDO, and soluble factors secreted by activated CD4⁺ T cells might contribute to IDO secretion by UC-MSCs.

Key words: Sjögren’s syndrome, mesenchymal stem cell, T follicular helper cell, indoleamine 2, 3-dioxygenase.

Introduction

Primary SS (pSS) is a chronic autoimmune disorder characterized by lymphocytic infiltration of salivary and lacrimal glands, leading to xerostomia and xerophthalmia. The prominent immune abnormalities in this disease are B cell overactivation that results in mass autoantibody production [1]. Also, germinal centre (GC)-like structures have been identified in the salivary glands of pSS patients [2], the presence of which correlates with the severity of inflammation and increased anti-Ro/SSA and anti-La/SSB autoantibody production [3].
T follicular helper (Tfh) cells are a specialized CD4+ T cell subset that typically expresses CXC chemokine receptor 5 (CXCR5), inducible T cell co-stimulator (ICOS), programmed cell death protein 1 (PD-1), transcriptional repressor B cell lymphoma 6 (Bcl-6) and IL-21 [4–6]. They have been recognized as essential for B cell maturation and immunoglobulin production [7]. CD4+CXCR5+ T cells in the blood, together with surface expression of ICOS and PD-1 alone or in combination, are generally accepted as circulating Tfh (cTfh) cells [8, 9]. Recent studies have shown that salivary gland epithelial cells from pSS patients are able to induce Tfh cell differentiation directly [10]. In addition, increased cTfh cell frequency has been reported in pSS and correlates with serum autoantibody levels and disease severity [11, 12], indicating that Tfh cells may play a part in the pathogenesis of this disease.

Mesenchymal stem cells (MSCs) are pluripotent stem cells with the capacity to differentiate into different cell lineages, such as osteoblasts, chondrocytes etc. [13, 14]. Furthermore, MSCs may modulate T and B lymphocytes, dendritic cells and natural killer cells, which makes them a promising alternative cell therapy for various autoimmune diseases, including SLE [15–18], RA [19, 20] and pSS [21]. We have previously shown the effectiveness and safety of allogeneic infusion of MSCs for refractory pSS [21]. Intriguingly, infusion of MSCs into non-obese diabetic (NOD) mice could significantly decrease the percentage of splenic CD4+CXCR5+ Tfh cells [21]. However, whether MSCs exert their therapeutic effect partly by inhibiting Tfh cells in pSS patients remains unclear. The aim of our study was to explore the effect of umbilical cord MSCs (UC-MSCs) on cTfh cells and the underlying mechanisms.

Materials and methods

Patients and controls

Blood samples were collected from pSS patients admitted to the Department of Immunology and Rheumatology, Drum Tower Clinical Medical College of Nanjing Medical University. UC-MSCs were prepared as described previously [18]. All UC-MSCs used in the experiment were derived from passages 3–5. Flow cytometric analysis showed CD29, CD44 and CD105 expression >95% in parallel with CD45, CD34, CD14 and HLA-DR expression <2%.

Co-culture of UC-MSCs with peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density-gradient centrifugation. UC-MSCs were co-cultured with 1 × 10^6 PBMCs isolated from pSS patients or HCs stimulated with phytohaemagglutinin (PHA; 2 µg/ml; Sigma, St Louis, MO, USA) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) for 3 days. In a trans-well system, UC-MSCs were cultivated in the lower chamber and PBMCs were seeded into the trans-well membrane of the inner chamber with 0.4-µm pore size (Corning, Corning, NY, USA).

Differentiation assay

Purified naive CD4+ T cells were isolated from PBMCs according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). UC-MSCs were co-cultured with 1 × 10^6 naive CD4+ T cells for 4 days at a ratio of 1:10 under Tfh cell-polarizing conditions: 3 µg/ml soluble anti-CD3 (eBioscience, San Diego, CA, USA), 3 µg/ml anti-CD28 (eBioscience), 50 ng/ml recombinant IL-6 (rIL-6; PeproTech, Rocky Hill, NJ, USA), 50 ng/ml rIL-21 (Abcam, Cambridge, MA, USA), 20 ng/ml rIL-12 (BioLegend, San Diego, CA, USA), 10 µg/ml anti-IL-4 antibody (eBioscience) and 10 µg/ml anti-IFN-γ antibody (eBioscience). An inhibitor of indoleamine 2,3-dioxygenase (IDO), 1-methyl-DL-tryptophan (1-MT; 100 µM; Sigma) or anti-IL-10 antibody (10 µg/ml; eBioscience) was added to the UC-MSC and Tfh cell differentiation co-culture system. To detect which factors involved in UC-MSC-mediated suppression, UC-MSCs were stimulated with PD-1 Fc (20 ng/ml; BioLegend) for 2 days and UC-MSCs were co-cultured with naive CD4+ T cells under Tfh cell-polarizing conditions or with anti-PDL1 antibody (10 µg/ml; BioLegend) for 2 days. To estimate whether pro-inflammatory mediators had an effect on the suppressive capacity of UC-MSCs, UC-MSCs were pre-stimulated with IFN-α (20 ng/ml; PeproTech, Rocky Hill, NJ, USA) or polyinosinic-polycytidylic acid (polyI:C; 100 µg/ml; Sigma) or lipopolysaccharide (LPS; 1 µg/ml; Sigma) for 24 h and

<table>
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<th>Characteristics</th>
<th>Values</th>
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<tr>
<td>Age, mean (±SEM), years</td>
<td>52.13 (11.23)</td>
</tr>
<tr>
<td>Men/women, n</td>
<td>8/60</td>
</tr>
<tr>
<td>Disease duration, mean (±SEM), years</td>
<td>6.25 (2.30)</td>
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<tr>
<td>Anti-Ro/SSA positive, %</td>
<td>65.24</td>
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<tr>
<td>Anti-La/SSB positive, %</td>
<td>30.32</td>
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Table 1 Clinical characteristics of 68 primary SS patients
then co-cultured with naive CD4+ T cells under Tfh cell-polarizing conditions for 4 days.

**Proliferation and apoptosis assay**

Purified CD4+ T cells were isolated from PBMCs according to the manufacturer’s instructions (Miltenyi). CD4+ T cells were labelled with 5 µM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA) in PBS for 10 min at 37°C. An excess of ice-cold RPMI 1640 with 10% FBS was added to the cells to quench the reaction. UC-MSCs were co-cultured with CFSE-labelled CD4+ T cells (1 x 10^5/well) for 4 days at a ratio of 1:10 with stimulation of 3 µg/ml soluble anti-CD3/CD28. Tfh cell proliferation was determined by flow cytometric analysis of CFSE fluorescence intensity. To detect apoptotic cells, cultured CD4+ T cells were resuspended in 100 µl of 1× binding buffer [10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), 140 mM NaCl and 2.5 mM CaCl2] and stained with 5 µl of FITC-conjugated annexin V (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions. For real-time PCR, cDNA was synthesized from TRIzol-isolated total RNA by use of the SuperScript III First Strand Synthesis SuperMix according to the manufacturer’s instructions. For real-time PCR experiments, reactions containing SYBR Premix EX Taq (Takara), ROX Reference Dye (50×; Takara), cDNA and gene primers were run on a StepOnePlus Real Time PCR System and analysed with StepOne software (version 2.1; Applied Biosystems, Carlsbad, CA, USA). The primers for different genes were:

- **GAPDH**: 5′-GACCGCTCAAGGCCTAGAAC-3′ (forward) and 5′-TGTTGAAGACCAGCGTGAAG-3′ (reverse);
- **Bcl-6**: 5′-GTTTCCGGACCTTACAGCT-3′ (forward) and 5′-CTGCCCTTGTGGAGCCATG-3′ (reverse);
- **T-bet**: 5′-AACATCTGTAGGGCCTGTTG-3′ (forward) and 5′-CCACCTGTGGTCTCAAGT-3′ (reverse);
- **GATA3**: 5′-TTACTCCCTCAGAGTGTGG-3′ (forward) and 5′-AAGTAACGGGACAGACCG-3′ (reverse);
- **ROA**: 5′-TCCACACAGCTTCTCCGG-3′ (forward) and 5′-TCCACAGATCCTTCTGATGA-3′ (reverse);
- **IL-21**: 5′-CATGGGAGAGATTGTACATTG-3′ (forward) and 5′-CCAGAATTCCGGGACCATGTC-3′ (reverse);
- **IDO**: 5′-GAATGGCACACGCTATGGAA-3′ (forward) and 5′-CACTCTCTATGAGACAGACAGTGC-3′ (reverse);
- **TGF-β**: 5′-AGCGAATGTCCAGAGTGATT-3′ (forward) and 5′-GCAGTGTGTTATCCCTGCTGTCA-3′ (reverse);
- **IL-10**: 5′-GAGATGCCTTCAGCAGAGTGAAGA-3′ (forward) and 5′-GGAGATGGCTATGGATGTCCTCAG-3′ (reverse);

**Flow cytometric analysis**

The cell suspension was labelled with the following monoclonal antibodies: FITC- or phycoerythrin (PE)-conjugated anti-CD4, Alexa Fluor 647-conjugated anti-CXCR5 and PerCP-Cy5.5-conjugated anti-PD-1 (BD). For surface marker staining, cells were maintained in the dark at 4°C for 30 min. For intracellular staining, cell suspension was incubated for 15 min in the dark at 4°C with surface antibody. After permeabilization, cell suspension was incubated for 30 min in the dark at 4°C with PE-conjugated anti-Foxp3 (eBioscience).

**Quantitative real-time PCR**

cDNA was synthesized from TRIzol-isolated total RNA by use of the SuperScript III First Strand Synthesis SuperMix for quantitative real-time PCR (Takara Bio, Otsu, Japan) according to the manufacturer’s instructions. For real-time PCR experiments, reactions containing SYBR Premix EX Taq (Takara), ROX Reference Dye (50×; Takara), cDNA and gene primers were run on a StepOnePlus Real Time PCR System and analysed with StepOne software (version 2.1; Applied Biosystems, Carlsbad, CA, USA). The primers for different genes were:

- **GAPDH**: 5′-GACCGCTCAAGGCCTAGAAC-3′ (forward) and 5′-TGTTGAAGACCAGCGTGAAG-3′ (reverse);
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- **T-bet**: 5′-AACATCTGTAGGGCCTGTTG-3′ (forward) and 5′-CCACCTGTGGTCTCAAGT-3′ (reverse);
- **GATA3**: 5′-TTACTCCCTCAGAGTGTGG-3′ (forward) and 5′-AAGTAACGGGACAGACCG-3′ (reverse);
- **ROA**: 5′-TCCACACAGCTTCTCCGG-3′ (forward) and 5′-TCCACAGATCCTTCTGATGA-3′ (reverse);
- **IL-21**: 5′-CATGGGAGAGATTGTACATTG-3′ (forward) and 5′-CCAGAATTCCGGGACCATGTC-3′ (reverse);
- **IDO**: 5′-GAATGGCACACGCTATGGAA-3′ (forward) and 5′-CACTCTCTATGAGACAGACAGTGC-3′ (reverse);
- **TGF-β**: 5′-AGCGAATGTCCAGAGTGATT-3′ (forward) and 5′-GCAGTGTGTTATCCCTGCTGTCA-3′ (reverse);
- **IL-10**: 5′-GAGATGCCTTCAGCAGAGTGAAGA-3′ (forward) and 5′-GGAGATGGCTATGGATGTCCTCAG-3′ (reverse);

**Determination of anti-Ro/SSA and anti-La/SSB using ELISA**

Anti-Ro/SSA and anti-La/SSB autoantibodies were determined by ELISA (Euroimmun, Morris Plains, NJ, USA). The sensitivity of the ELISA kits was 1 RU/ml.

**Determination of IL-10 using the Luminex cytokine assay**

The IL-10 level in the supernatant was measured with a Cytokine Human Magnetic 7-Plex Panel assay (Millipore, Billerica, MA, USA) on a Luminex 100 system (Luminex, Austin, TX, USA) according to the manufacturer’s instructions. The sensitivity was 1 pg/ml.

**Statistical analysis**

Data were summarized as mean (±S.E.M.). Statistical significance was determined by Student’s t-test and the correlation coefficient between Tfh cell concentration and clinical features were analysed by Spearman’s rank test. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). A P-value < 0.05 was considered significantly significant.

**Results**

Positive correlation of cTfh cells with serum anti-La/SSB level and ESSDAI in pSS patients

First, we examined peripheral cTfh cell frequency in pSS patients and HCs. Flow cytometric analysis showed that
the percentage of CD4+CXCR5+PD-1+ cTfh cells was significantly up-regulated in pSS patients [6.55% (S.E.M. 0.51) vs 2.32% (0.13), P < 0.01; Fig. 1A]. Next, correlations between cTfh cell frequency and ESSDAI or autoantibody level were analysed. Among the 48 pSS patients, cTfh cell frequency was positively correlated with ESSDAI (r = 0.64, P < 0.001). In those patients with serum anti-Ro/SSA and La/SSB levels >2 RU/ml, cTfh cell frequency had a positive correlation with serum anti-La/SSB level (r = 0.50, P = 0.02), but not with anti-Ro/SSA level (r = 0.20, P = 0.20; Fig. 1B). Anti-La/SSB is a more specific autoantibody for pSS patients and is related to disease activity and severity [24], suggesting that enhanced cTfh cell frequency promoted the development of pSS, possibly by stimulating anti-La/SSB autoantibody production.

UC-MSCs inhibited the generation of cTfh cells in vitro

In order to test the immunomodulatory role of UC-MSCs in cTfh cells, PBMCs from both pSS patients and HCs were co-cultured with UC-MSCs. Fig. 2A showed that UC-MSCs dose-dependently suppressed cTfh cell generation in pSS patients [38.38% (S.E.M. 3.58) vs 17.81% (3.53), P < 0.05; Fig. 2A], but up-regulated CXCR5+CD4+ T cell frequency (see supplementary Fig. S1A, available at Rheumatology Online). Furthermore, UC-MSCs could markedly inhibit cTfh cell generation in HCs [29.86% (S.E.M. 4.13) vs 19.73% (2.80), P < 0.01; Fig. 2B] as in pSS patients [38.38% (S.E.M. 3.58) vs 17.81% (3.53), P < 0.05]. However, UC-MSCs did not inhibit the generation of CXCR5+CD4+ T cells in pSS patients or HCs (see supplementary Fig. S1B, available at Rheumatology Online), suggesting that UC-MSCs down-regulated cTfh cell production in a trans-well system. We found that there were no significant differences in inhibition of cTfh cell generation by UC-MSCs between cell-to-cell contact and the trans-well system, suggesting that soluble factors secreted by UC-MSCs may play an important role in suppressing cTfh cell generation [38.38% (S.E.M. 3.58) vs 17.81% (3.53) vs 23.01% (3.07), P < 0.05; Fig. 2C).

UC-MSCs inhibited the differentiation and proliferation of cTfh cells

Since UC-MSCs suppressed the generation of cTfh cells in pSS patients, we were interested in ascertaining whether this inhibitory effect was caused by affecting cTfh differentiation, proliferation or apoptosis. First, naive CD4+ T cells isolated from pSS patients were
induced to differentiate into Tfh cells. Supplementary Fig. S2A, available at Rheumatology Online, shows that the mRNA expression of IL-21 and Bcl-6 (the specific transcription factor for Tfh cells) was up-regulated and these differentiated T cells expressed ICOS, CXCR5 and PD-1 (supplementary Fig. S2B, available at Rheumatology Online). This evidence showed these induced CXCR5+ T cells to be Tfh cells. Then we co-cultured UC-MSCs with naive CD4+ T cells under Tfh cell-polarizing conditions. The results showed that the percentage of CD4+CXCR5+PD-1+ T cells significantly decreased in the presence of UC-MSCs both in pSS patients (24.02% (S.E.M. 3.91) vs 6.37% (3.28), \( P < 0.05 \), *P < 0.01. pSS: primary SS; UC-MSCs: umbilical cord mesenchymal stem cells; cTfh: circulating T follicular helper; pSS: primary SS; PBMCs: peripheral blood mononuclear cells; HCs: healthy controls.

However, UC-MSCs did not influence the expression of Foxp3 on CXCR5+ T cells [5.42% (S.E.M. 1.22) vs 6.22% (2.21), \( P > 0.05 \)], but did up-regulate the expression on CXCR5+ T cells [3.38% (S.E.M. 0.63) vs 4.93% (0.71), \( P < 0.05 \), *Fig. 3D], suggesting that Tfr cells are not involved in UC-MSC-mediated Tfh cell inhibition.

UC-MSCs exerted their suppressive function on cTfh cells through the secretion of IDO

We tried to identify which soluble factors were responsible for the inhibitory effect of UC-MSCs on cTfh cells in pSS patients. We measured the mRNA levels of IDO, IL-10, COX-2, HGF and TGF-\( \beta \) expressed by UC-MSCs in the UC-MSC and cTfh cell differentiation co-culture system. The results showed that UC-MSCs expressed a much higher level of IDO mRNA when co-cultured with naive CD4+ T cells under Tfh cell-polarizing conditions compared with UC-MSCs cultured alone or with Tfh cell-inducing factors [0.78 (S.E.M. 0.19) vs 5.14 (1.28), \( P < 0.05 \); Fig. 4A]. In addition, IDO mRNA expression by UC-MSCs was increased when UC-MSCs were co-cultured with anti-CD3/CD28-activated CD4+ T cells both by cell-to-cell contact and in a trans-well system (see supplementary Fig. S3A and B, available at Rheumatology Online), although IDO expression was markedly lower compared with UC-MSCs cultured alone or with Tfh cell-inducing factors [0.78 (S.E.M. 0.19) vs 5.14 (1.28) vs 31 046 (15 228), \( P < 0.05 \); Fig. 4A].

In addition, IDO mRNA expression by UC-MSCs was increased when UC-MSCs were co-cultured with anti-CD3/CD28-activated CD4+ T cells both by cell-to-cell contact and in a trans-well system (see supplementary Fig. S3A and B, available at Rheumatology Online), although IDO expression was markedly lower compared with UC-MSCs co-cultured with naive CD4+ T cells under Tfh cell-polarizing conditions. However, IDO expression by UC-MSCs was extremely decreased in the co-culture system of UC-MSCs and naive CD4+ T cells, suggesting that the significantly higher expression of IDO could be induced only when UC-MSCs were co-cultured with naive CD4+ T cells.
with activated CD4⁺ T cells. We also found that IDO expression by UC-MSCs was only a little higher when UC-MSCs were stimulated with PD-1 (see supplementary Fig. S3C, available at *Rheumatology* Online). Consistently, the addition of anti-PDL1 antibody to the UC-MSCs and cTfh cell differentiation co-culture system had no effect on the secretion of IDO (see supplementary Fig. S3D, available at *Rheumatology* Online). These results indicate that UC-MSCs inhibit Tfh cell differentiation via soluble factors, but not the interaction of surface molecules.

Our study also showed increased IL-10 mRNA levels in UC-MSCs co-cultured with naive CD4⁺ T cells under Tfh cell-polarizing conditions [0.33 (S.E.M. 0.12) vs 0.67 (0.35) vs 670.7 (435.1), P < 0.05], but not COX-2/prostaglandin E2 (PGE2) [0.95 (S.E.M. 0.07) vs 0.62 (0.22) vs 0.72 (0.60), P > 0.05], HGF [0.93 (S.E.M. 0.19) vs 0.98 (0.30) vs 1.45 (0.51), P > 0.05] or TGF-β [0.90 (S.E.M. ± 0.12) vs 0.55 (0.09) vs 1.98 (0.34), P > 0.05; Fig. 4A]. HPLC results further exhibited a higher level of IDO enzymatic activity in the supernatant of the UC-MSC and Tfh cell differentiation co-culture group through measuring the level of kynurenine [1.74 µM (S.E.M. 0.59) vs 2.92 (0.78) vs 43.14 (5.15), P < 0.05 or P < 0.01; Fig. 4B]. The level of IL-10 in the supernatant of the co-culture system was also increased [0.24 pg/ml (S.E.M. 0.03) vs 0.60 (0.08) vs 126.50 (60.22), P < 0.05; Fig. 4C]. These results indicated that IDO and IL-10, especially IDO, might be important factors in UC-MSC-mediated suppression. To further confirm this, IDO inhibitor 1-MT or anti-IL-10 antibody was added to the co-culture system. As Fig. 5A shows, 1-MT partly reversed the immunosuppressive effect of UC-MSCs on the differentiation of cTfh cells [2.52% (S.E.M. 3.63) vs 9.15 (3.03) vs 17.72 (3.69), P < 0.05], whereas anti-IL-10 antibody did not have this effect [24.73% (S.E.M. 2.13) vs 15.45 (2.00) vs 15.38 (2.79), P > 0.05; Fig. 5B]. Therefore this suggests that IDO might be the major factor in the UC-MSC-mediated suppressive function.

Reportedly Toll-like receptor 3 (TLR3) and TLR4 are overexpressed in the labial salivary glands of pSS patients, especially in infiltrating mononuclear cells, acinar cells and ductal epithelial cells [26]. pSS patients also display their own IFN signature and immune complexes induce the production of IFN-α in pSS patients [27]. Therefore we investigated whether the ability of UC-MSCs to suppress Tfh cell differentiation was affected in the presence of such pro-inflammatory mediators as IFN-α, poly(I:C) (TLR3 ligand) or LPS (TLR4 ligand). The results demonstrated that there were no differences in

(A) UC-MSCs significantly inhibited the differentiation of cTfh cells in both pSS patients (n = 5) and HCs (n = 4). (B) UC-MSCs significantly inhibited the proliferation of cTfh cells in both pSS patients (n = 4) and HCs (n = 5). (C) UC-MSCs had no effect on the apoptosis of cTfh cells in pSS patients (n = 6) or HCs (n = 6). (D) Foxp3 expression on both CXCR5⁺ and CXCR5⁻ T cells when naive CD4⁺ T cells were co-cultured with UC-MSCs under Tfh cell-polarizing conditions in pSS patients (n = 7). *P < 0.05, **P < 0.01. UC-MSCs: umbilical cord mesenchymal stem cells; cTfh: circulating T follicular helper; pSS: primary SS; HCs: healthy controls.
suppressing Tfh cell differentiation by prestimulating UC-MSCs with either IFN-α, polyI:C or LPS [17,10% (S.E.M. 2.36) vs 7.15 (1.07) vs 7.44 (0.20) vs 7.78 (0.20) vs 6.23 (0.42), P > 0.05; Fig. 5C]. Also, IDO mRNA expression on UC-MSCs did not change after stimulation with IFN-α, polyI:C or LPS [2.68 (S.E.M. 0.62) vs 5.17 (1.85) vs 1.33 (0.07) vs 0.93 (0.24), P > 0.05; Fig. 5D].

Discussion

Tfh cells play an important role in the pathogenesis of various autoimmune diseases by assisting B cells in the production of high-affinity autoantibodies. Our present study showed a significantly up-regulated CD4⁺CXCR5⁺PD-1⁺ cTfh cell percentage in pSS patients and this increased percentage of cTfh cells was positively correlated with elevated levels of anti-La/SSB and ESSDAI. Anti-La/SSB is more relevant to organ involvement in pSS patients [24], suggesting that cTfh cells in pSS patients might promote the development of this disease by helping B cells with the production of higher levels of anti-La/SSB.

Current therapy for pSS is based mainly upon corticosteroids and immunosuppressants according to the degree of organ damage. However, long-term application of these conventional drugs could lead to severe adverse
effects, including infection, diabetes, osteoporosis and caput femoris necrosis. Therefore the search for high-efficiency and low side-effect therapeutic approaches has been the focus of research. In recent years MSCs have attracted widespread attention and been regarded as a novel cell therapy for autoimmune diseases because of their immunomodulatory capacity and lower immunogenicity. We previously infused MSCs into 24 refractory pSS patients and found that all patients’ symptoms improved after MSC transplantation, concomitant with reduced levels of serum anti-Ro/SSA and anti-La/SSB antibodies and decreased percentage of cTfh cell [21], indicating that MSCs might exert their therapeutic efficacy partly by modulating cTfh cells. Here we verified that UC-MSCs could dose-dependently inhibit the generation of cTfh cells in pSS patients but up-regulate the percentage of CXCR5⁺CD4⁺ T cells. UC-MSCs have been reported to balance Th1/Th2 and Th17/Treg cells [28], which indicates that UC-MSCs down-regulate the frequency of cTfh cells, possibly through the balance of CXCR5⁺ and CXCR5⁻ T cells. Moreover, we found that UC-MSCs suppressed cTfh cell generation via secretion of soluble factors in vitro, and we recognized that this reduced cTfh cell generation was ascribed to the inhibition of UC-MSCs in the differentiation of these cells.

Tfr cells are a new type of Treg that share similar phenotypic characteristics with Tfh cells but express Foxp3, originating from natural Treg cells [29]. It has been reported that Tfh cells in the blood promote antibody production, whereas Tfr cells strongly inhibit antibody production in mice [30]. In addition, Tfr cells may limit the GC reaction by Tfh cells and the amount of antigen-specific IgM, IgG1, IgG2b and IgA [29, 31]. This evidence indicates that Tfr cells might control the function of Tfh cells. However, in our study we did not find that UC-MSCs could up-regulate the expression of Foxp3 on CXCR5⁺ T cells, suggesting that Tfr cells are not involved in UC-MSC-mediated Tfh cell inhibition.

Many studies have begun to elucidate what factors govern the immune state of MSCs. IDO, nitric oxide and interleukins have been attributed to the immunosuppressive effect of MSCs [32–36]. Recent data have shown that MSCs can inhibit allogeneic T cell responses in mixed lymphocyte reactions in an IDO-dependent manner [37]. In our experiment, we found that the mRNA expression of IDO in the UC-MSC co-culture system was greatly

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**Fig. 5** Inhibition of Tfh cell differentiation was mediated by the secretion of IDO on UC-MSCs

(A) 1-MT was added to the UC-MSC and Tfh cell differentiation co-culture system (n = 5). (B) Anti-IL-10 antibody was added to the UC-MSC and Tfh cell differentiation co-culture system (n = 4). (C) UC-MSCs were prestimulated with IFN-α, poly(I:C) or LPS for 24 h and then co-cultured with naive CD4⁺ T cells under Tfh cell-polarizing conditions in pSS patients (n = 3). (D) mRNA expression of IDO by UC-MSCs co-cultured with naive CD4⁺ T cells under Tfh cell-polarizing conditions in pSS patients (n = 3). *P < 0.05, **P < 0.01. Tfh: circulating T follicular helper; IDO: indoleamine 2,3-dioxygenase; UC-MSCs: umbilical cord mesenchymal stem cells; poly(I:C): polyinosinic-polycytidylic acid; LPS: lipopolysaccharide; pSS: primary SS.
increased and was accompanied by a higher level of IDO enzymatic activity in the supernatant, indicating that IDO is the critical factor responsible for the suppressive function of MSCs on cTfh cells. This notion was confirmed by the addition of IDO inhibitor 1-MT, which reversed the inhibitory effect of UC-MSCs on Tfh cells.

IDO is a ubiquitously expressed intracellular enzyme that degrades the essential amino acid tryptophan in mammals, catalysing the initial and rate-limiting step in the de novo biosynthesis of nicotinamide adenine dinucleotide [38]. Increased expression of IDO has been shown in MSCs following exposure to IFN-γ [39, 40], which was identified as the main mechanism of T cell suppression by human MSCs [39]. IDO may be involved in many aspects of MSC-mediated immune suppression, including a direct effect on T cells by tryptophan depletion or toxic metabolites of tryptophan [41–43]; an indirect effect through altered function of antigen-presenting cells, especially dendritic cells [44, 45]; inducing the generation of Tregs [46, 47] and eliciting the conversion of monocytes into IL-10-secreting M2 immunosuppressive macrophages [48].

Taken together, our findings suggest that UC-MSCs could reduce the generation of cTfh cells in pSS patients by inhibiting cTfh cell differentiation through the secretion of IDO. Our study provides support for the application of MSCs in some autoimmune diseases associated with cTfh cell abnormality and sheds light on the development of novel therapeutic approaches targeting cTfh cells. However, further studies are still needed to clarify the roles of cTfh cells in pSS patients in vivo and the exact mechanisms of IDO suppression on cTfh cells.

**Rheumatology key messages**

- Circulating Tfh cells might be involved in the pathogenesis of primary SS.
- Umbilical cord mesenchymal stem cells exhibited a therapeutic effect on primary SS patients by inhibiting circulating Tfh cell generation.
- The suppression of primary SS circulating Tfh cell differentiation by umbilical cord mesenchymal stem cells might be associated with IDO expression.

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**Supplementary data**

Supplementary data are available at *Rheumatology* Online.

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