RHEUMATOLOGY

Rheumatology 2015;54:332–342
doi:10.1093/rheumatology/keu316
Advance Access publication 27 August 2014

Original article

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 Liu[1], Dinglei Su[1], Min Zhou[1], Xuebing Feng[1], Xia Li[2] and Lingyun Sun[1]

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 $CXCR5^+PD\text{-}1^+CD4^+$ 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. Naive $CD4^+$ T cells were isolated from PBMCs and then co-cultured with UC-MSCs under Tfh cell-polarizing conditions. The percentage of $CXCR5^+PD\text{-}1^+CD4^+$ 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 naive $CD4^+$ 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 naive $CD4^+$ T cells in a trans-well system. In addition, HPLC showed increased IDO enzymic activity in the supernatant of UC-MSCs co-cultured with naive $CD4^+$ 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.

BASIC SCIENCE

Introduction

Primary SS (pSS) is a chronic autoimmune disorder characterized by lymphocytic infiltration of salivary and lachrymal 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].

[1]Department of Immunology and Rheumatology, Drum Tower Clinical Medical College of Nanjing Medical University, Nanjing, Jiangsu and [2]Department of Immunology, College of Basic Medical Science, Dalian Medical University, Dalian, Liaoning, People's Republic of China.

Submitted 18 November 2013; revised version accepted 19 June 2014.

Correspondence to: Lingyun Sun, Department of Immunology and Rheumatology, Drum Tower Clinical Medical College of Nanjing Medical University, 321 Zhongshan Road, Nanjing 210008, China. E-mail: lingyunsun2012@163.com

Rui Liu and Dinglei Su contributed equally to this study.

Xia Li and Lingyun Sun contributed equally to this study.

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. All patients fulfilled the American–European Consensus Group criteria for pSS diagnosis [22] and they had no other autoimmune or systemic diseases. The European League Against Rheumatism (EULAR) SS Disease Activity Index (ESSDAI) score was assessed [23] at the time we examined the frequency of cTfh cells in pSS patients. Blood samples from age- and sex-matched healthy controls (HCs) were obtained from the Medical Examination Center of Drum Tower Clinical Medical College of Nanjing Medical University. This research was in compliance with the Declaration of Helsinki. The study was approved by the ethics committee of Drum Tower Clinical Medical College of Nanjing Medical University. All patients and HCs were informed and consented to this study. Detailed clinical characteristics are shown in Table 1.

Table 1 Clinical characteristics of 68 primary SS patients

Characteristics	Values
Age, mean (S.E.M.), years	52.13 (11.23)
Men/women, *n*	8/60
Disease duration, mean (S.E.M.), years	6.25 (2.30)
Anti-Ro/SSA positive, %	65.24
Anti-La/SSB positive, %	30.32

Isolation and culture of UC-MSCs

Fresh human umbilical cords were obtained from 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 prestimulated 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

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 (1×10^6/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 $CaCl_2$) and stained with 5 μl of FITC-conjugated annexin V (BD Biosciences, San Diego, CA, USA) and then analysed by flow cytometry.

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.

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 listed:

GAPDH: 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGTGAAGACGCCAGTGGA-3′ (reverse);
Bcl-6: 5′-GTTTCCGGCACCTTCAGACT-3′ (forward) and 5′-CTGGCTTTTGTGACGGAAAT-3′ (reverse);
T-bet: 5′-AACATCCTGTAGTGGCTGGTG′ (forward) and 5′-CCACCTGTTGTGGTCCAAGT-3′ (reverse);
GATA3: 5′-TTCCTCCTCCAGAGTGTGGT′ (forward) and 5′-AAAATGAACGGACAGAACCG-3′ (reverse);
RORA: 5′-TCTCCCTGCGCTCTCCGCAC′ (forward) and 5′-TCCACAGATCTTGCATGGA′ (reverse);
IL-21: 5′-CATGGAGAGGATTGTCATCTGTC′ (forward) and 5′-CAGAAATTCAGGGACCAAGTCAT′ (reverse);
IDO: 5′-GAATGGCACACGCTATGGAA-3′ (forward) and 5′-CAGACTCTATGAGATCAGGCAGATG-3′ (reverse);
hepatocyte growth factor (HGF): 5′-GTCAGCCCTGGAGT TCCATGATA-3′ (forward) and 5′-AGCGTACCTCTGGAT TGCTTGTG-3′ (reverse);
TGF-β: 5′-AGCGACTCGCCAGAGTGGTTA-3′ (forward) and 5′-GCAGTGTGTTATCCCTGCTGTCA-3′ (reverse);
IL-10: 5′-GAGATGCCTTCAGCAGAGTGAAGA-3′ (forward) and 5′-AGTTCACATGCGCCTTGATGTC-3′ (reverse);
cyclooxygenase 2 (COX-2): 5′-TGACCAGAGCAGGCAGA TGAA-3′ (forward) and 5′-CCACAGCATCGATGTCACCA TAG-3′ (reverse).

IDO activity assay

Kynurenine metabolites were estimated by reverse-phase HPLC. In brief, 400 μl of culture supernatant was diluted with 400 μl of potassium phosphate buffer (0.05 M, pH 6.0) and protein was precipitated with 100 μl of 2 M trichloroacetic acid. Three hundred microlitres of supernatant was then injected into an RP18 column and eluted with a degassed potassium phosphate solution (0.015 M, pH 6.4) containing 27 ml/l acetonitrile at a flow rate of 0.5 ml/min. Kynurenine was detected by an ultraviolet (UV) detector at a wavelength of 350 nm. Tryptophan concentration was measured using a fluorescence detector at an excitation wavelength of 284 nm and an emission wavelength of 365 nm. The values were referred to a standard curve with defined kynurenine and tryptophan concentrations (0–60 μM).

Statistical analysis

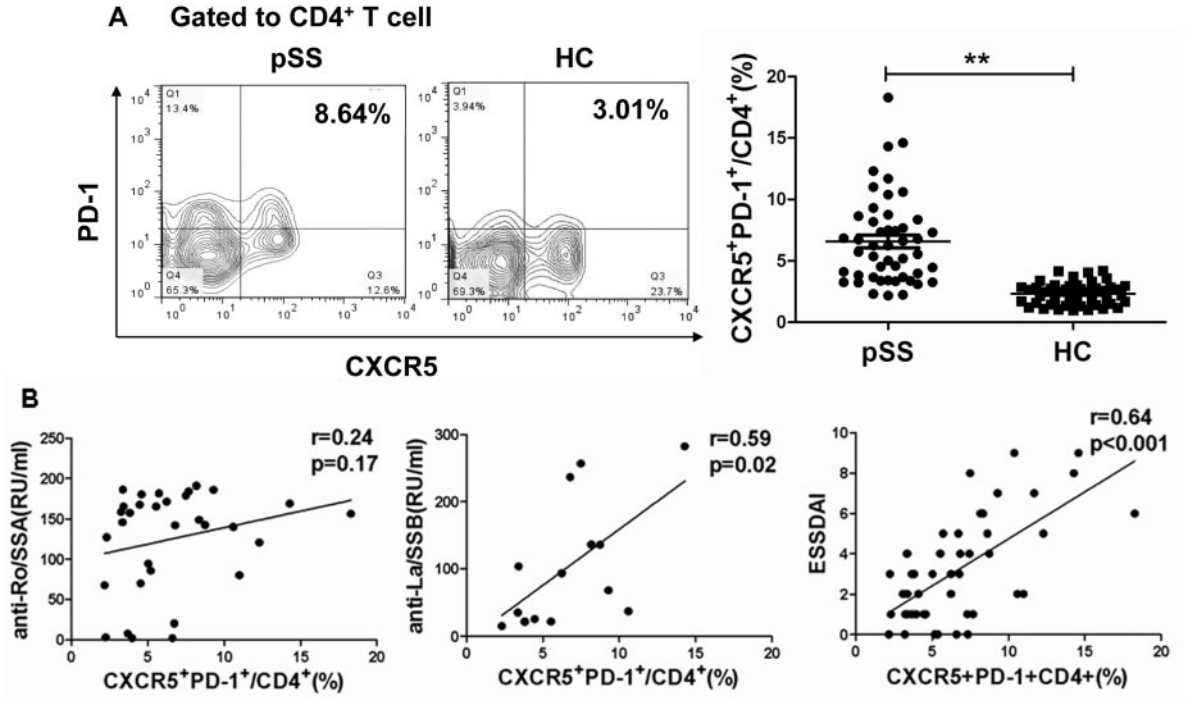
Data were summarized as mean (S.E.M.). Statistical significance was determined by Student's *t*-test and the correlation coefficient between Tfh cell concentration and clinical features were analysed by Spearman's rank test. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad 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

FIG. 1 Elevated frequency of cTfh cells and its correlation with serum levels of anti-La/SSB and ESSDAI in pSS patients



(**A**) Percentage of $CD4^+CXCR5^+PD\text{-}1^+$ cTfh cells in pSS patients ($n = 48$) and HCs ($n = 47$). (**B**) Correlations of the percentage of $CD4^+CXCR5^+PD\text{-}1^+$ cTfh cell frequency with serum levels of anti-Ro/SSA, anti-La/SSB and ESSDAI in pSS patients. $^*P < 0.05$, $^{**}P < 0.01$. cTfh: circulating T follicular helper; ESSDAI: European League Against Rheumatism (EULAR) SS Disease Activity Index; pSS: primary SS; HC: healthy controls.

the percentage of $CD4^+CXCR5^+PD\text{-}1^+$ 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.

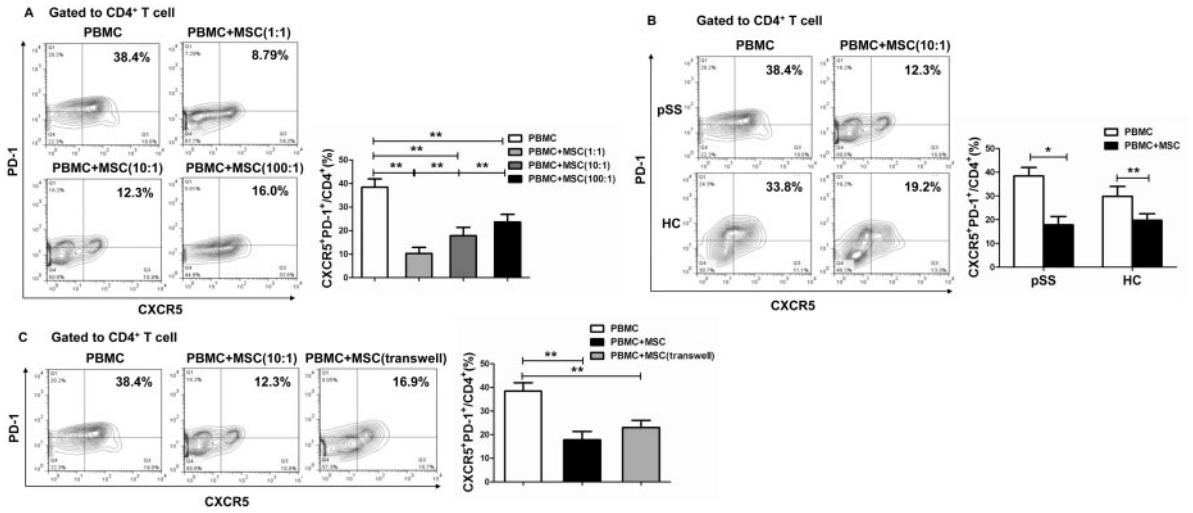
UC-MSCs inhibited the generation of cTfh cells *in vitro*

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* 10.31% (2.54) *vs* 17.81% (3.53) *vs* 23.63% (3.23), $P < 0.01$; 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 frequency, possibly through the balance of $CXCR5^+$ and $CXCR5^-$ T cells. To explore the mechanism of UC-MSC-mediated immune suppression, PBMCs were co-cultured with UC-MSCs 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

FIG. 2 UC-MSCs suppressed cTfh cell generation through the secretion of soluble factors



(**A**) UC-MSCs dose-dependently suppressed cTfh cell generation in pSS patients ($n = 6$). (**B**) The percentage of cTfh cells was down-regulated when UC-MSCs were co-cultured with activated PBMCs isolated from both pSS patients ($n = 6$) and HCs ($n = 5$). (**C**) UC-MSCs suppressed peripheral cTfh cell generation both in a cell-to-cell contact and a trans-well system in pSS patients ($n = 6$). $^*P < 0.05$, $^{**}P < 0.01$. 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.

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 $CD4^+CXCR5^+PD\text{-}1^+$ 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), $P < 0.05$] and HCs [21.73% (S.E.M. 1.92) *vs* 14.15% (1.63), $P < 0.01$; Fig. 3A]. CFSE-labelled $CD4^+$ T cells were co-cultured with UC-MSCs. We found that the proliferative rate of $CD4^+CXCR5^+PD\text{-}1^+$ T cells was substantially attenuated in the UC-MSC co-culture group both in pSS patients [51.90% (S.E.M. 2.46) *vs* 8.90% (0.32), $P < 0.01$] and HCs [52.56% (S.E.M. 1.28) *vs* 23.45% (8.67), $P < 0.05$; Fig. 3B]. Unexpectedly, we did not find an obvious effect of UC-MSCs on the apoptosis of cTfh cells either in pSS patients [4.18% (S.E.M. 0.92) *vs* 5.49% (1.17), $P > 0.05$] or HCs [1.89% (S.E.M. 0.25) *vs* 2.84% (0.51), $P > 0.05$; Fig. 3C].

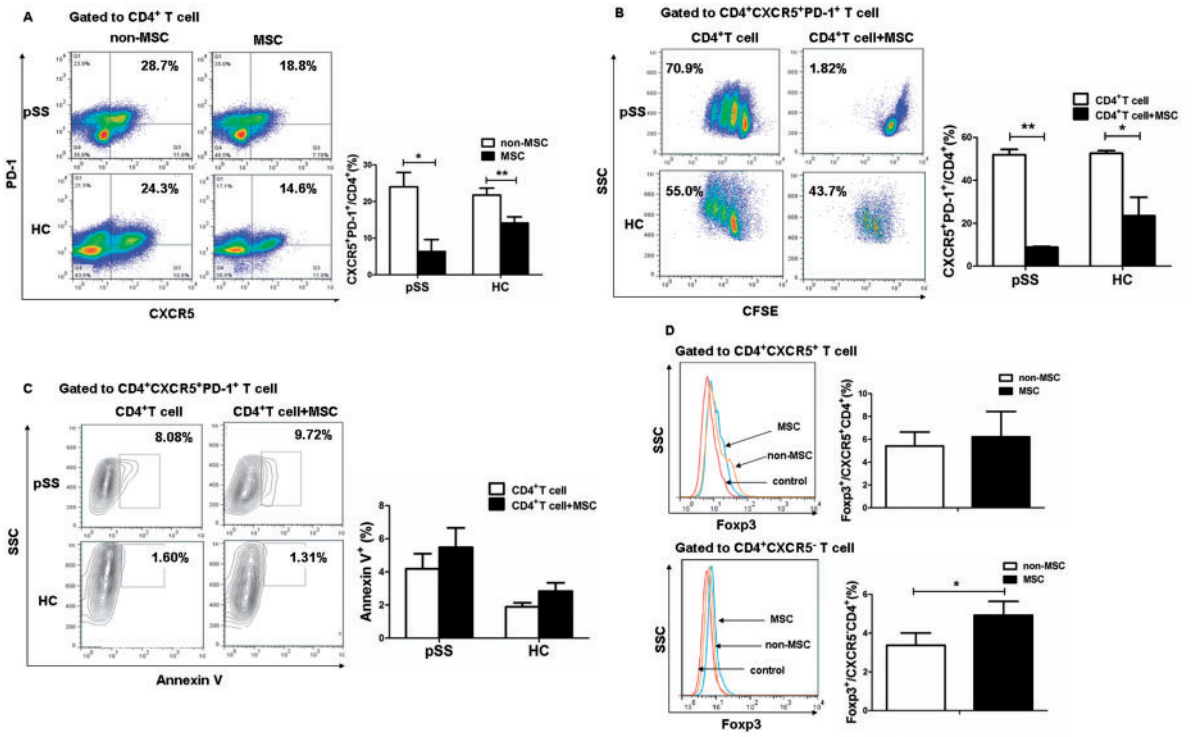
Follicular regulatory T (Tfr) cells are a type of follicular T cell, with phenotypic characteristics of Tfh cells but expressing Foxp3. Reportedly ~5–25% of Tfh cells are $Foxp3^+$ Tfr cells [25]. Tfr cells can limit the magnitude of the GC reaction and the production of immunoglobulin. We next detected whether UC-MSCs were able to up-regulate the proportion of Tfr cells, which subsequently inhibited Tfh cells in the process of Tfh cell differentiation. 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-β 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) *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

FIG. 3 UC-MSCs inhibited the differentiation and proliferation of cTfh cells in pSS patients



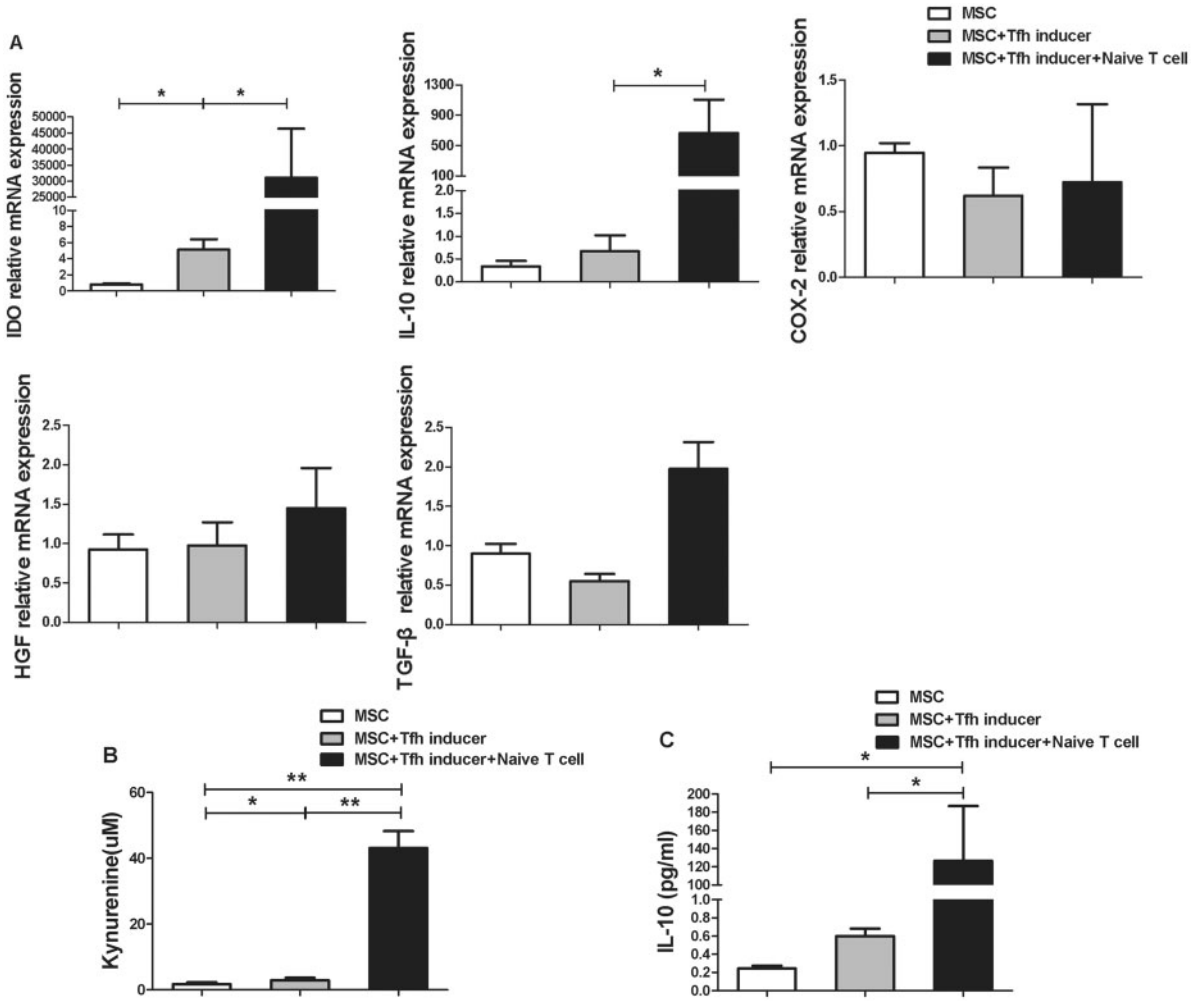
(**A**) UC-MSCs significantly inhibited the differentiation of cTfh cells in both pSS patients ($n = 5$) and HCs ($n = 4$). (**B**) UC-MSCs significantly inhibited the proliferation of cTfh cells in both pSS patients ($n = 4$) and HCs ($n = 5$). (**C**) UC-MSCs had no effect on the apoptosis of cTfh 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.

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, polyI:C (TLR3 ligand) or LPS (TLR4 ligand). The results demonstrated that there were no differences in

FIG. 4 mRNA expression of various factors secreted by UC-MSCs



(A) mRNA expression of IDO, IL-10, COX-2, HGF and TGF-β by UC-MSCs cultured alone or with Tfh cell-inducing factors or with naive $CD4^+$ T cells under Tfh cell-polarizing conditions in pSS patients ($n = 4$). (**B**) Functional IDO activity by UC-MSCs cultured alone or with Tfh cell-inducing factors or with naive T cells under Tfh cell-polarizing conditions was measured in terms of the ability to metabolize tryptophan to kynurenine ($n = 6$). (**C**) The level of IL-10 in the supernatant of UC-MSCs cultured alone or with Tfh cell-inducing factors or with naive $CD4^+$ T cells under Tfh cell-polarizing conditions in pSS patients ($n = 6$). pSS: primary SS; UC-MSCs: umbilical cord mesenchymal stem cells; IDO: indoleamine 2,3-dioxygenase; COX-2: cyclooxygenase 2; HGF: hepatocyte growth factor; Tfh: T follicular helper.

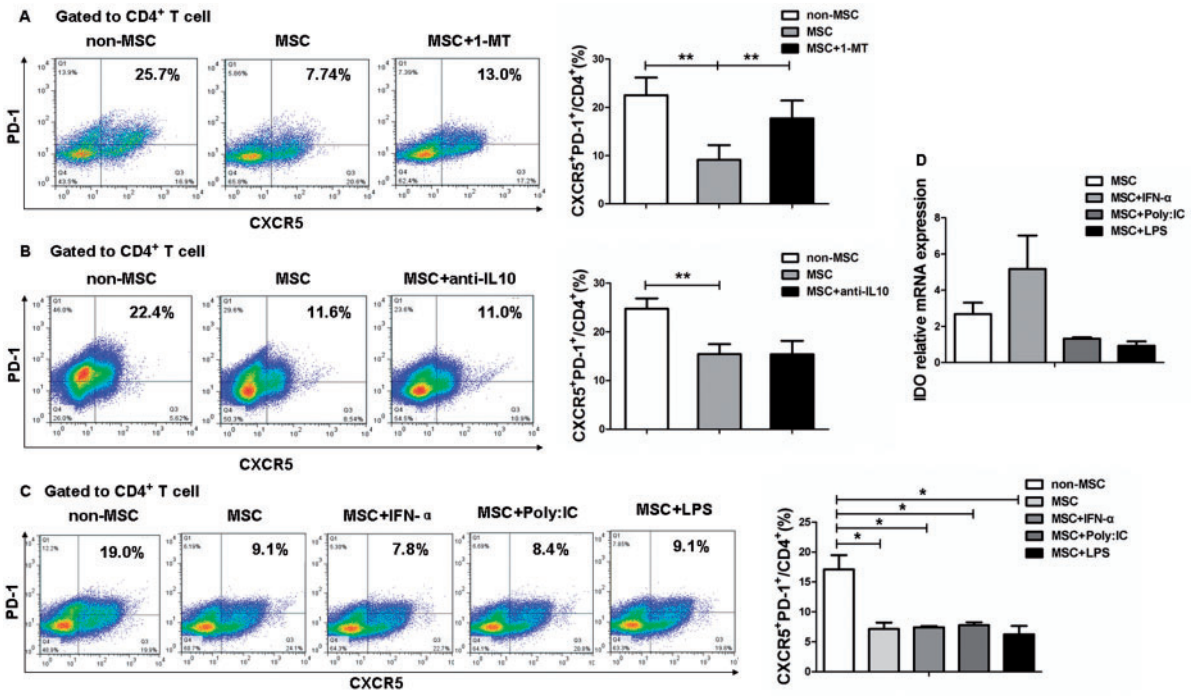
suppressing Tfh cell differentiation by prestimulating UC-MSCs with either IFN-α, polyI:C or LPS [17.10% (S.E.M. 2.36) *vs* 7.15 (1.07) *vs* 7.44 (0.20) *vs* 7.78 (0.47) *vs* 6.23 (0.42), $P > 0.05$; Fig. 5C]. Also, IDO mRNA expression on UC-MSCs did not change after stimulation with IFN-α, polyI:C or 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 $CD4^+CXCR5^+$ $PD\text{-}1^+$ 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

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, polyI: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; polyI:C: polyinosinic–polycytidylic acid; LPS: lipopolysaccharide; pSS: primary SS.

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

increased and was accompanied by a higher level of IDO enzymic 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.

Funding: This work was supported by the Major International (Regional) Joint Research Project (81120108021), National Natural Science Foundation of China (81172847 and 81373214), Jiangsu Province Kejiao Xingwei Program, China Postdoctoral Science Foundation (First Class; 2012M510073).

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

References

1 Fox RI. Sjogren's syndrome. Lancet 2005;366:321–31.

2 Amft N, Curnow SJ, Scheel-Toellner D *et al*. Ectopic expression of the B cell-attracting chemokine BCA-1 (CXCL13) on endothelial cells and within lymphoid follicles contributes to the establishment of germinal center-like structures in Sjogren's syndrome. Arthritis Rheum 2001; 44:2633–41.

3 Hansen A, Lipsky PE, Dorner T. B cells in Sjogren's syndrome: indications for disturbed selection and differentiation in ectopic lymphoid tissue. Arthritis Res Ther 2007;9: 218.

4 Rasheed AU, Rahn HP, Sallusto F, Lipp M, Muller G. Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. Eur J Immunol 2006;36:1892–903.

5 Haynes NM, Allen CD, Lesley R *et al*. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. J Immunol 2007;179: 5099–108.

6 Kroenke MA, Eto D, Locci M *et al*. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. J Immunol 2012;188:3734–44.

7 Breitfeld D, Ohl L, Kremmer E *et al*. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med 2000;192:1545–52.

8 Simpson N, Gatenby PA, Wilson A *et al*. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis Rheum 2010;62: 234–44.

9 Morita R, Schmitt N, Bentebibel SE *et al*. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity 2011;34:108–21.

10 Gong YZ, Nititham J, Taylor K *et al*. Differentiation of follicular helper T cells by salivary gland epithelial cells in primary Sjogren's syndrome. J Autoimmun 2014;51: 57–66.

11 Li XY, Wu ZB, Ding J *et al*. Role of the frequency of blood CD4(+)CXCR5(+)CCR6(+) T cells in autoimmunity in patients with Sjogren's syndrome. Biochem Biophys Res Commun 2012;422:238–44.

12 Szabo K, Papp G, Barath S *et al*. Follicular helper T cells may play an important role in the severity of primary Sjogren's syndrome. Clin Immunol 2013;147:95–104.

13 Pittenger MF, Mackay AM, Beck SC *et al*. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.

14 Sanchez-Ramos J, Song S, Cardozo-Pelaez F *et al*. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 2000;164:247–56.

15 Gu Z, Akiyama K, Ma X *et al*. Transplantation of umbilical cord mesenchymal stem cells alleviates lupus nephritis in MRL/lpr mice. Lupus 2010;19:1502–14.

16 Zhou K, Zhang H, Jin O *et al*. Transplantation of human bone marrow mesenchymal stem cell ameliorates the

autoimmune pathogenesis in MRL/lpr mice. Cell Mol Immunol 2008;5:417–24.

17 Sun L, Akiyama K, Zhang H *et al*. Mesenchymal stem cell transplantation reverses multiorgan dysfunction in systemic lupus erythematosus mice and humans. Stem Cells 2009;27:1421–32.

18 Sun L, Wang D, Liang J *et al*. Umbilical cord mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus. Arthritis Rheum 2010;62:2467–75.

19 Augello A, Tasso R, Negrini SM, Cancedda R, Pennesi G. Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. Arthritis Rheum 2007;56:1175–86.

20 Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. Arthritis Rheum 2009;60:1006–19.

21 Xu J, Wang D, Liu D *et al*. Allogeneic mesenchymal stem cell treatment alleviates experimental and clinical Sjogren syndrome. Blood 2012;120:3142–51.

22 Vitali C, Bombardieri S, Jonsson R *et al*. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554–8.

23 Seror R, Ravaud P, Bowman SJ *et al*. EULAR Sjogren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjogren's syndrome. Ann Rheum Dis 2010;69:1103–9.

24 Locht H, Pelck R, Manthorpe R. Clinical manifestations correlated to the prevalence of autoantibodies in a large (n=321) cohort of patients with primary Sjogren's syndrome: a comparison of patients initially diagnosed according to the Copenhagen classification criteria with the American-European consensus criteria. Autoimmun Rev 2005;4:276–81.

25 Linterman MA, Pierson W, Lee SK *et al*. Foxp3+ follicular regulatory T cells control the germinal center response. Nat Med 2011;17:975–82.

26 Kawakami A, Nakashima K, Tamai M *et al*. Toll-like receptor in salivary glands from patients with Sjogren's syndrome: functional analysis by human salivary gland cell line. J Rheumatol 2007;34:1019–26.

27 Lovgren T, Eloranta ML, Kastner B *et al*. Induction of interferon-alpha by immune complexes or liposomes containing systemic lupus erythematosus autoantigen- and Sjogren's syndrome autoantigen-associated RNA. Arthritis Rheum 2006;54:1917–27.

28 Liu Y, Mu R, Wang S *et al*. Therapeutic potential of human umbilical cord mesenchymal stem cells in the treatment of rheumatoid arthritis. Arthritis Res Ther 2010;12:R210.

29 Wollenberg I, Agua-Doce A, Hernandez A *et al*. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. J Immunol 2011;187:4553–60.

30 Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. Nat Immunol 2013;14:152–61.

31 Crotty S. Follicular helper CD4 T cells (TFH). Annu Rev Immunol 2011;29:621–63.

32 Spaggiari GM, Capobianco A, Abdelrazik H *et al*. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood 2008;111:1327–33.

33 Nemeth K, Leelahavanichkul A, Yuen PS *et al*. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med 2009;15:42–9.

34 Neuss S, Becher E, Woltje M, Tietze L, Jahnen-Dechent W. Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair, and wound healing. Stem Cells 2004;22:405–14.

35 Jui HY, Lin CH, Hsu WT *et al*. Autologous mesenchymal stem cells prevent transplant arteriosclerosis by enhancing local expression of interleukin-10, interferon-γ, and indoleamine 2,3-dioxygenase. Cell Transplant 2012;21:971–84.

36 Patel SA, Meyer JR, Greco SJ *et al*. Mesenchymal stem cells protect breast cancer cells through regulatory T cells: role of mesenchymal stem cell-derived TGF-β. J Immunol 2010;184:5885–94.

37 Meisel R, Zibert A, Laryea M *et al*. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004;103:4619–21.

38 Grohmann U, Fallarino F, Puccetti P. Tolerance, DCs and tryptophan: much ado about IDO. Trends Immunol 2003;24:242–8.

39 Ren G, Su J, Zhang L *et al*. Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. Stem Cells 2009;27:1954–62.

40 Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-γ does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol 2007;149:353–63.

41 Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? Immunol Today 1999;20:469–73.

42 Frumento G, Rotondo R, Tonetti M *et al*. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. J Exp Med 2002;196:459–68.

43 Terness P, Bauer TM, Rose L *et al*. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. J Exp Med 2002;196:447–57.

44 Von Bubnoff D, Scheler M, Wilms H, Fimmers R, Bieber T. Identification of IDO-positive and IDO-negative human dendritic cells after activation by various proinflammatory stimuli. J Immunol 2011;186:6701–9.

45 Harden JL, Egilmez NK. Indoleamine 2,3-dioxygenase and dendritic cell tolerogenicity. Immunol Invest 2012;41:738–64.

46 Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J Immunol 2008;181:5396–404.

47 Chung DJ, Rossi M, Romano E *et al*. Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. Blood 2009;114:555–63.

48 Francois M, Romieu-Mourez R, Li M, Galipeau J. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. Mol Ther 2012;20:187–95.