Mesenchymal stem cells negatively regulate CD4+ T cell activation in patients with primary Sjögren syndrome through the miRNA-125b and miRNA-155 TCR pathway

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Abstract. Treatment with mesenchymal stem cells (MSCs) has been revealed to suppress CD4+ T cells and autoimmunity in both mouse models and patients with primary Sjögren syndrome (pSS); however, the underlying mechanism remains unclear. MicroRNAs (miRNAs or miRs) mediate CD4+ T cell activation, but the mechanism is not understood, particularly for CD4+ T cells treated with MSCs. Characterization of miRNAs may reveal pSS pathogenesis, guide MSC treatment and provide more personalized management options. The present study aimed to perform an miRNome analysis of quiescent and T cell receptor (TCR)-activated CD4+ T cells treated with MSCs via miRNA profiles and bioinformatics. Following 72 h of co-culture, MSCs inhibited TCR-induced CD4+ T cell activation and decreased IFN-γ levels. The numbers of aberrant miRNAs in pSS naïve (vs. healthy naïve), pSS activation (vs. pSS naïve), MSC treatment and pre-IFN-γ MSC treatment (vs. pSS activation) groups were 42, 55, 27 and 32, respectively. Gene enrichment analysis revealed that 259 pathways were associated with CD4+ T cell stimulation, and 240 pathways were associated with MSC treatment. Increased miRNA-7150 and miRNA-5096 and decreased miRNA-125b-5p and miRNA-22-3p levels in activated CD4+ T cells from patients with pSS were reversed by MSC treatment. Notably, the proliferation of CD4+ T cells and CD4+ IFN-γ+ cells, expression levels of miRNA-125b-5p and miRNA-155 in CD4+ T cells and supernatant IFN-γ secretion were associated with disease activity. miRNA may play a vital role in MSC treatment for activated CD4+ T cells. The results indicated that the expression levels of miRNA-125b-5p and miRNA-155 in TCR-activated CD4+ T cells from patients with pSS may provide insight regarding autoimmune diseases and offer a novel target for prospective treatment. Therefore, these results may be crucial in providing MSC treatment for pSS.

Introduction

Primary Sjögren syndrome (pSS) is an autoimmune disease that can attack the exocrine glands, causing symptoms such as xerostomia and keratoconjunctivitis sicca (1). However, treatments for patients with pSS, such as stimulating drugs and artificial saliva, are ineffective and only symptomatic (2).

Mesenchymal stem cells (MSCs), such as human umbilical cord mesenchymal stem cells (hUCMSCs), offer a promising treatment for pSS due to their low immunogenicity and immunoregulatory potential (3). MSCs have been reported to exert inhibitory functions on activated lymphoid cells, including CD4+ T cells (4). However, the underlying mechanisms, such as direct cell contact and secretion of soluble mediators, including prostaglandin E2 (PGE2), IL-10, TGF-β and hepatic growth factor (5), are contradictory. However, the regulatory mechanisms underlying CD4+ T cell activation by MSCs are still unclear due to their multiplicity, for example, subtle gene regulation. The microRNA (miRNA or miR) pathway may be involved in gene regulation for CD4+ T cell activation (6).

miRNAs have been reported to control T cell activation (6). miRNA microarray has been used to identify unique miRNAs associated with glandular inflammation and dysfunction

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Abbreviations: MSCs, mesenchymal stem cells; pSS, primary Sjögren syndrome; GO, Gene Ontology; PGE2, prostaglandin E2; IDO, indoleamine 2,3-dioxygenase; UCs, umbilical cords; KEGG, Kyoto Encyclopedia of Genes and Genomes

Key words: CD4+ T cell, mesenchymal stem cells, microRNA, primary Sjögren syndrome, T cell receptor pathway

from patients with pSS (7). Furthermore, pathway analysis of miRNAs predicted to target Ro/SSA and La/SSB autoantigens revealed differential miRNA expression levels in the salivary glands and peripheral blood mononuclear cells (PBMCs) from patients with pSS (7).

In light of miRNA function in CD4^{+} T cells and pSS pathogenesis, MSCs may exert immunomodulatory effects on CD4^{+} T cells and offer a potential treatment for pSS. Therefore, the present study aimed to perform an miRNome analysis of quiescent and T cell receptor (TCR)-activated CD4^{+} T cells treated with MSCs via miRNA profiling and bioinformatics. The interaction between miRNAs and regulatory pathways (particularly the TCR pathway) was studied in TCR-activated CD4^{+} T cells to provide a novel understanding of pSS progression and MSC treatment mechanisms.

Materials and methods

Isolation, cultivation, immunophenotyping and labeling of hUCMSCs. The present study was approved (approval no. K-2012-006) by the Research Ethics Committee of Tongji Hospital of Tongji University (Shanghai, China). hUCMSCs were isolated from full-term infants after obtaining parental written consent (8). Briefly, Wharton's jelly tissue was separated from UCs, digested with 1 mg/ml collagenase type I (Sigma-Aldrich; Merck KGaA) and then plated in fresh culture medium. Following expansion for 1 week, adherent cells were obtained and replated in DMEM (Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO_2.

At passage 4, hUCMSCs were identified by flow cytometry with a FACSCalibur II flow cytometer (BD Biosciences), using antibodies (all from eBioscience; Thermo Fisher Scientific, Inc.) against MSCs [CD13-APC (cat. no. 47-0138-42), CD54-APC (cat. no. 17-0542-82), CD73-PE (cat. no. 25-0739-42), CD166-PE (cat. no. 12-1668-42) and CD90-APC (cat. no. 47-0909-41)], hematopoietic cells [CD14-FITC (cat. no. MHCD1401), CD19-FITC (cat. no. 11-0193-82), CD34-PE (cat. no. 12-0349-41), CD45-PE-Cy7 (cat. no. MHCD4512) and CD117-APC (cat. no. 47-1171-80)], integrins [CD29-APC (cat. no. 17-0291-80)], extracellular matrix receptors [CD44-FITC (cat. no. 11-0441-86)] and major histocompatibility complexes [HLA-DR-PerCP (cat. no. 46-9952-41) and HLA-ABC-FITC (cat. no. 11-9983-41)]. MSCs were stained with primary human albumin-FITC (cat. no. CLFAG2140; Cedarlane Laboratories) and pan-cytokeratin-FITC (cat. no. 130-119-141; Miltenyi Biotec GmbH), and then with secondary FITC rabbit anti-human albumin (cat. no. A18904; eBioscience; Thermo Fisher Scientific, Inc.). MSCs were inductively cultured to assay adipogenic, osteoplastic and chondrogenic differentiation to assess their multipotency, as previously described (9).

Patients and controls. Venous blood was collected from inpatients with pSS at the Department of Rheumatology and Immunology, Tongji Hospital of Tongji University (Shanghai, China) between January 2013 and December 2016. The pSS diagnosis complied with the American-European Consensus Group criteria (10). The patients had no other autoimmune diseases and took no immunosuppressive drugs. Healthy controls (HCs) were recruited from the Examination Department, Tongji Hospital of Tongji University. The present

study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients and HCs. Clinical features are presented in Table I. The pSS activity was evaluated using the EULAR Sjögren's syndrome disease activity index (ESSDAI) (11).

Peripheral CD4^{+} T cell separation. Venous blood was collected in EDTA tubes for PBMC isolation within 4 h using Ficoll-Hypaque density configuration (Sigma-Aldrich; Merck KGaA). CD4^{+} T cells stained at 4°C for 20 min with FITC anti-human CD4 (cat. no. 300506; BioLegend, Inc.) were sorted on a FACSCalibur flow cytometer.

CD4^{+} T cell and MSC co-culture experiments. Following isolation, the CD4^{+} T cells were divided into five groups: Healthy naïve (CD4^{+} T cells from HC), pSS naïve (CD4^{+} T cells from patients with pSS), pSS activation [CD4^{+} T cells from patients with pSS stimulated by anti-CD3 antibody and anti-CD28 antibody (BioLegend, Inc.) for 72 h], MSC treatment (stimulated CD4^{+} T cells from patients with pSS co-cultured with MSCs for 72 h) and pre-IFN-γ MSC treatment [stimulated CD4^{+} T cells from patients with pSS co-cultured with MSCs (pre-stimulated by IFN-γ) for 72 h]. CD4^{+} T cell proliferation was analyzed using flow cytometry with CellTrace™ CFSE cell proliferation kit (cat. no. C34554; Thermo Fisher Scientific, Inc.). The CFSE plot consisted of certain characteristic ridges demonstrating cell proliferation following stimulation. CD4^{+} T cell division was denoted by the mean generation number (MGN). Cells were gated in compliance with their forward- and side-scatter characteristics for the purpose of excluding dead cells and debris. For flow cytometry, primary antibodies [CD4-PE (cat. no. 565999) and IFN-γ-FITC (cat. no. 561057; both from BD Biosciences)] were added to the cells at 4°C for 20 min. The cells were operated on a FACS Calibur and studied using CellQuest™ Pro software (BD Biosciences). The co-culture supernatants were tested by ELISA, according to the manufacturer's instructions (Shanghai Westang Bio-Tech).

miRNA microarray. The microarray assay was performed using a facilitator (LC Sciences). The 3' end of the micromolecular RNAs (4 μg) was elongated by adding a poly (A) tail and ligated with pCp-Cy3 dyes. Hybridization was carried out at 4°C for 20 h on a μParaflo microfluidic array. Following RNA hybridization, fluorescence signals were scanned using a laser scanner (GenePix 4000B; Molecular Devices LLC), analyzed with Array-Pro image analysis software version X3 (Media Cybernetics, Inc.) and then standardized with a locally weighted scatterplot smoothing filter as previously described (12).

Reverse transcription-quantitative (RT-q)PCR. The miRNAs were confirmed via stem-loop RT-PCR. Total RNA from CD4^{+} T cells was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. cDNA was synthesized from 0.5 μg RNA, using a reverse transcription kit (Takara Bio, Inc.), according to the manufacturer's protocols. miRNAs were reverse-transcribed using a specific primer (Table SI). qPCR was run with a specific primer (Table SI) and SYBR Premix Ex Taq (Takara Bio, Inc.) on an ABI PRISM 7500 Real-Time PCR system

Table I. Clinical characteristics of patients with pSS and healthy controls.

Clinical characteristics	Patients with pSS, n=13	Healthy controls, n=13
Age, years (mean ± SEM)	19-68 (47.21±8.33)	20-65 (48.42±9.75)
Female, %	100	100
Mean disease duration, years (SEM)	6.09 (3.54)	-
Anti-SSA positive, %	100	0
Anti-SSB positive, %	100	0
Antinuclear antibody positive, %	100	0
Lymphocytic focus score ≥1 foci, %	100	-
IgG >16 g/l, %	100	0

pSS, primary Sjögren syndrome.

Table II. MSCs inhibit mitogenic CD4^{+} T cell proliferation.

MGN statistics	pSS activation	MSC treatment (MSC:CD4^{+} T)			Pre-IFN-γ MSC treatment (MSC:CD4^{+} T)		
		1:5	1:10	1:20	1:5	1:10	1:20
MGN	6.62±1.28	2.89±0.53	3.36±0.69	5.97±0.81	3.14±0.78	3.79±0.46	6.58±0.95
q-value	-	3.67	6.33	2.09	4.27	5.99	1.76
P-value	-	<0.05[a]	<0.01[b]	>0.05[c]	<0.01[a]	<0.01[b]	>0.05[c]

CFSE-labeled CD4^{+} T cells were activated by CD3/CD28 agonists and co-cultured with different MSC concentrations for 72 h. CD4^{+} T cell inhibition, as revealed by decreased MGN, was dependent on MSC:T cell ratio. MSC induced significant inhibition of T cell proliferation at high ratios (1:5 and 1:10). n=8. [a]P vs. pSS activation; [b]P vs. 1:10; [c]P vs. 1:20. MSC, mesenchymal stem cells; pSS, primary Sjögren syndrome; MGN, mean generation number.

(Applied Biosciences; Thermo Fisher Scientific, Inc.). PCR was performed as follows: 95°C for 10 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing/extension at 60°C for 20 sec. The relative changes were calculated using the $2^{-\Delta\Delta Cq}$ method (13); healthy naïve individuals were used as the control.

Pathway and miRNA gene network. Two databases [TargetScan (targetscan.org/) and miRanda (microrna.org)] were used to predict the combined target genes of aberrant miRNAs in the two groups. Gene Ontology (GO) was applied to predict the primary function of target genes based on the microarray data obtained as aforementioned (14,15). A pathway study was used to identify the significant pathway of differential genes in accordance with Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta and Reactome analysis (15,16). Furthermore, Fisher's exact test was used to select the crucial pathway, and the ingate was determined using the P and false discovery rate values. The enrichment Re was calculated as described previously (17-19), which was as follows by: Re=(nf/n)/(Nf/N), where nf is the number of differential genes within the particular category, n is the total number of genes within the same category, while Nf is the number of differential genes in the entire microarray, and N is the total number of genes in the microarray. The association between miRNAs and targets was acquired based on differential levels and their associations in the Sanger miRBase Release 20.0 (sanger. ac.uk/Software/Rfam/mirna/) for miRNAs to construct an miRNA gene network (19).

Statistical analysis. Data were analyzed using SPSS 17.0 (SPSS, Inc.) for Windows, followed by two-tailed, unpaired or paired Student's t-test for significant differences. Data are presented as the mean ± SEM of three independent repeats. P<0.05 was considered to indicate a statistically significant difference.

Results

Isolation and identification of hUCMSCs. The primary hUCMSC culture took 5-10 days to reach sub-confluence. Flow cytometry revealed that the cells did not exhibit hematopoietic progenitor labels (for example, CD45, CD34, CD14 and HLA-DR) but expressed MSC markers such as CD73, CD105, CD166 and CD90 (data not shown). After 10 days, the attached cells were fibroblast-like. MSCs also differentiated into adipocytes, chondrocytes and osteocytes (data not shown).

MSCs inhibit proliferation of CD4^{+} T cells. Following co-culture for 72 h, MSCs suppressed CD3/CD28-stimulated CD4^{+} T cell multiplication under a dose-dependent mode, as determined by a decrease in the CFSE peak generation number (Table II). Under co-culture through cell-cell contact, MGN decreased in terms of MSC:CD4^{+} ratio (1:10 and 1:5; MSC
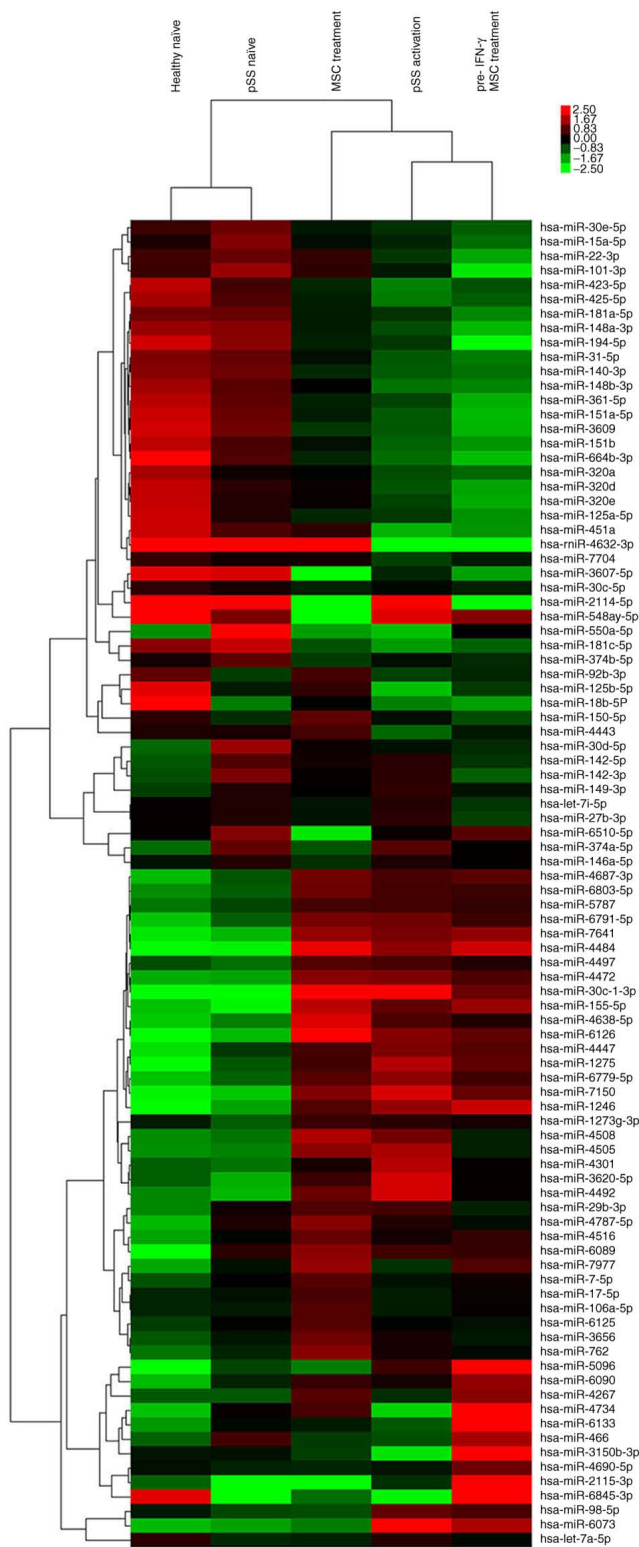
Figure 1. Expression level profiles of miRNAs in CD4⁺ T cells from healthy and pSS naïve, pSS activation, MSC treatment and pre-IFN-γ MSC treatment groups were detected using miRNA microarray. Top bar represents signal levels of miRNA expression from -2.5 (green) to +2.5 (red). Individual >2-fold dysregulated miRNAs are presented on the right. miRNA, microRNA; pSS, primary Sjögren syndrome; MSC, mesenchymal stem cell.

the present study, MSC pretreatment with IFN-γ did not result in increased CD4⁺ T cell inhibition.

Activated CD4⁺ T cells and MSC treatment demonstrate different miRNA signatures. A genome-wide survey of miRNA expression levels was performed using miRNA microarray for 2,578 human miRNA sequences enumerated in the Sanger database. After separating signals from noise, the numbers of distinct miRNAs in pSS naïve (vs. healthy naïve), pSS activation (vs. pSS naïve), MSC treatment (vs. pSS activation) and pre-IFN-γ MSC treatment (vs. MSC treatment) groups were 42, 55, 27 and 32, respectively (Fig. 1; Table SII). Compared with the pSS naïve group, 26 of 55 miRNAs were upregulated in the pSS activation group. The top 10 upregulated miRNAs were miRNA-30c-1-3p, -155-5p, -1246, -1273g-3p, -1275, -4472, -4638-5p, -5096, -7150 and -7641. The top down-regulated miRNAs were miRNA-15a-5p, -30d-5p, -30e-5p, -140-3p, -181a-5p, -451a, -3607-5p, -4443, -4734 and -6510-5p. miRNA-15a-5p, -30d-5p, -451a, -1246, -1275, -4734, -5096 and -6510-5p were also differentially expressed between pSS and healthy naïve groups (Fig. 1; Table SII). These data indicated that these miRNAs were involved in pSS pathogenesis.

Given the unique miRNA profiling in stimulated CD4⁺ T cells from patients with pSS, the effect of MSC treatment alone on miRNome patterns of activated CD4⁺ T cells was further investigated. Compared with pSS activation, 13 of 27 differentially expressed miRNAs were upregulated following MSC treatment (Fig. 1; Table SII), including miRNA-92b-3p, -125b-5p, -150-5p, -155-5p, -451a, -3150b-3p, -4267, -4443, -4484, -4638-5p, -4734, -6126 and -7977. The downregulated miRNAs were miRNA-98-5p, -146a-5p, -374a-5p, -1246, -1275, -3607-5p, -3620-5p, -4301, -4492, -5096, -6073, -6510-5p and -7150. Moreover, the upregulation of miRNA-98-5p, -1246, -1275, -3620-5p, -4301, -4492, -5096, -6073 and -7150 and miRNA-155-5p, -4484, -4638-5p and -6216 in pSS activation was reversed or promoted by MSC treatment, respectively. Downregulation of miRNA-125b-5p, -451a, -3150b-3p, -4443 and -4734 and miRNA-3607-5p and -6510-5p in pSS activation was reversed or promoted by MSC treatment, respectively.

Although MSC pretreatment by IFN-γ did not inhibit CD4⁺ T cell proliferation more potently compared with MSC treatment alone, 32 differentially expressed miRNAs existed between the two groups (Fig. 1; Table SII). The primary upregulated miRNAs in the pre-IFN-γ MSC treatment group included miRNA-146a-5p, -466, -1246, -3150b-3p, -4267, -4690-5p, -4734, -5096, -6090 and -6133, whereas the downregulated miRNAs included miRNA-22-3p, -30c-1-3p, -150-5p, -451a, -762, -3656, -4508, -4638-5p and -6126.

miRNA target prediction by two databases. In light of the impact of pSS activation and MSC treatment on CD4⁺ T cell proliferation, TargetScan and miRanda were used to predict the combined target genes of aberrant miRNAs in the two groups. A total of 3,124 and 2,127 target genes were predicted for the 55 miRNAs in the pSS activation group and 27 miRNAs in the MSC treatment group, respectively (Tables SIII and SIV).

miRNA action on activated CD4⁺ T cells and MSC treatment via bioinformatics. KEGG predicted that 55 miRNAs in the pSS activation group significantly upregulated 128 and

treatment vs. pSS activation). This confirmed previous studies describing dose-dependent MSC inhibition of T cell proliferation (20,21). IFN-γ has previously been reported to trigger the MSC inhibitory effects on T cell proliferation (22,23), but in
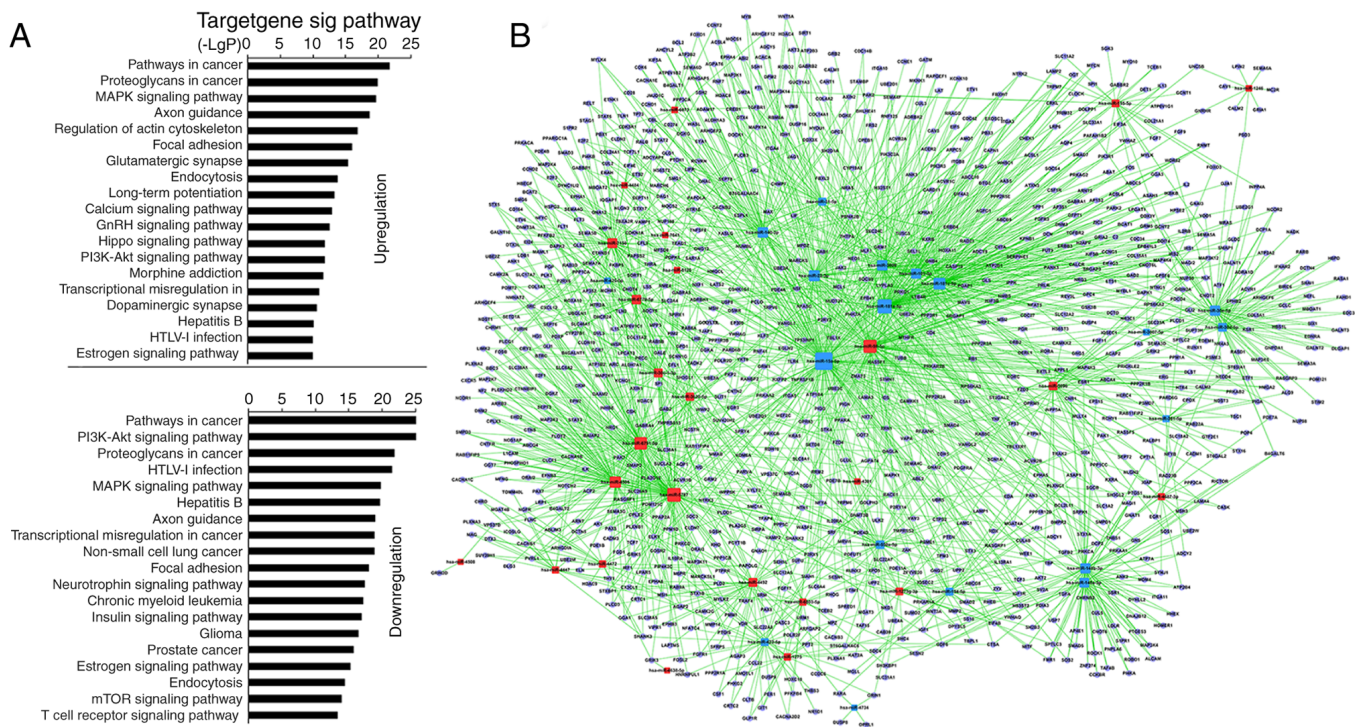
Figure 2. Prediction of signaling pathways and the mRNA-miRNA network in CD4+ T cells from the pSS activation group. (A) miRNA-Kyoto Encyclopedia of Genes and Genomes network. Vertical axis is the pathway category; horizontal axis is the P-value of each pathway. Lower P-values indicate more miRNAs regulate the pathways and the pathways serve more important roles in activated CD4+ T cells. (B) miRNA-mRNA-network. Red squares represent upregulated miRNAs; yellow squares represent downregulated miRNAs; blue circles represent genes; lines represent the association between the miRNA and the gene. The network is for the assessment of regulatory status of miRNAs and genes. The degree (size) of squares indicates regulatory functionality of miRNA (i.e. bigger degree indicates more functions). Similarly, the degree of the blue circle is consistent with the network linkage (bigger circle indicates greater regulation by miRNAs). miRNA, microRNA; pSS, primary Sjögren syndrome.

downregulated 131 GO terms (Table SV). Among the upregulated GOs, the top 10 were associated with 'pathways in cancer', 'proteoglycans in cancer', 'MARK signaling pathway', 'regulation of actin cytoskeleton', 'axon guidance', 'focal adhesion', 'glutamatergic synapse', 'endocytosis', 'long-term potentiation' and 'calcium signaling pathway' (Fig. 2A; Table SV). Among the downregulated GOs, the top 10 were associated with 'PI3K-AKT signaling pathway', 'pathways in cancer', 'HTLV-I infection', 'proteoglycans in cancer', 'hepatitis B', 'MAPK signaling pathway', 'axon guidance', 'non-small-cell lung cancer', 'transcriptional misregulation in cancer' and 'focal adhesion' (Fig. 2A; Table SV). 'TCR signaling pathway', which is stimulated directly by the anti-CD3 antibody and anti-CD28 antibody, was significantly downregulated (Fig. 2A; Table SV). The miRNA-mRNA network via bioinformatics predicted that the top 20 GO terms showing a high enrichment degree were upregulated by miRNA-5787, -98-5p, -6791-5p, -4505, -7150, -6779-5p, -30c-1-3p, -155-5p and -5096, and downregulated by miRNA-15a-5p, -181a-5p, -181c-5p, -22-3p, -140-3p, -3609, -30e-5p, -148b-3p, -101-3p, -148a-3p and -30d-5p (Fig. 2B and Table SVI). Notably, miRNA-155-5p, -98-5p, -5096, -5787, -181a-5p, -15a-5p, -148b-3p, -140-3p, -7150 and -3609 participated in 'TCR signaling pathway' (Table SVII). miRNA-155 was predicted to regulate the TCR signaling pathway via targeting key genes [such as Fos, p21 (RAC1) activated kinase (PAK)2, MAP3K14, and PIK3R1] whereas miRNA-181a-5p was predicted to target Fos and tumor necrosis factor. Other high-degree miRNAs were also associated with 'TCR signaling pathway' [miRNA-5096 and -148b-3p targeted SOS1; miRNA-15a-5p targeted KRAS; miRNA-7150 targeted 3-phosphoinositide-dependent protein kinase 1, MAP2K4 and P73; miRNA-98-5p targeted AKT2, CBL, RAS guanyl releasing protein 1 (RASGPR1), VAV3 and PAK1; Table SVII]. The microarray analysis indicated that miRNA-92b-3p, -125b-5p and -150-5p exhibited the highest upregulation, and miRNA-146a-5p, -374a-5p and -1246 exhibited the greatest downregulation. However, bioinformatics demonstrated that the aforementioned miRNAs were not involved in 'TCR signaling pathway'.

Analysis using KEGG revealed that 27 miRNAs in MSC-treated CD4+ T cells upregulated 117 and downregulated 123 GO terms significantly (Table SVIII). Among the upregulated GO terms, the top 10 were associated with 'proteoglycans in cancer', 'endocytosis', 'pathways in cancer', 'neurotrophin signaling pathway', 'HTLV-I infection', 'PI3K-AKT signaling pathway', 'MAPK signaling pathway', 'axon guidance', 'glioma' and 'regulation of actin cytoskeleton'. Among the downregulated GO terms, the top 10 were associated with 'proteoglycans in cancer', 'pathways in cancer', 'neurotrophin signaling pathway', 'PI3K-AKT signaling pathway', 'MAPK signaling pathway', 'axon guidance', 'regulation of actin cytoskeleton', 'Wnt signaling pathway', 'focal adhesion' and 'ErbB signaling pathway' (Fig. 3A; Table SVIII). Furthermore, GO terms, including 'proteoglycans in cancer', 'endocytosis, pathways in cancer', 'MAPK signaling pathway', 'HTLV-I infection', 'PI3K-AKT signaling pathway',
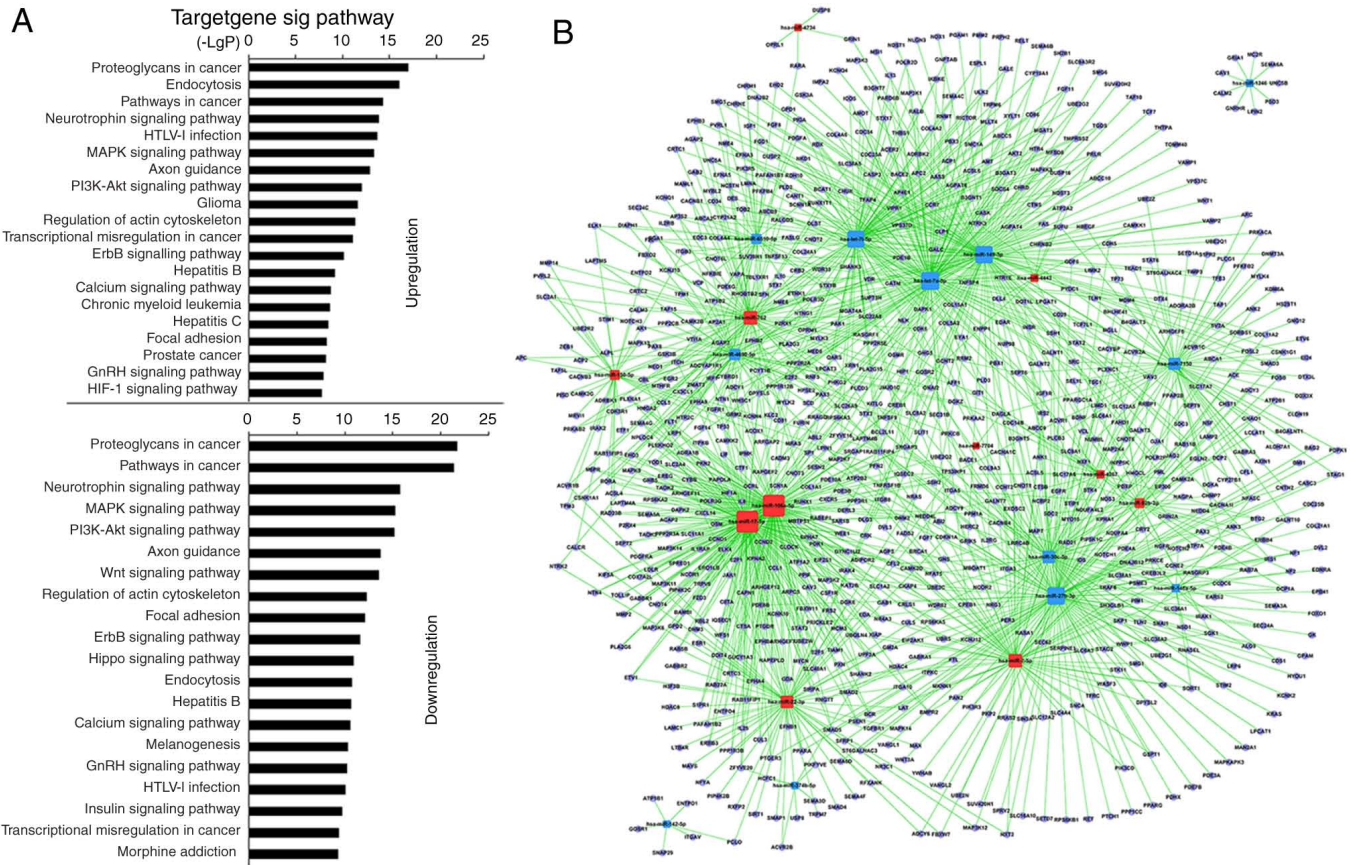
Figure 3. Prediction of signaling pathways and the mRNA-miRNA network in CD4+ T cells from MSC treatment group. (A) miRNA-Kyoto Encyclopedia of Genes and Genomes network. Horizontal axis is the P-value of each pathway. Lower P-values indicate more miRNAs regulate the pathways and the pathways serve more important roles in MSC-treated CD4+ T cells. (B) miRNA-mRNA-network. Red squares represent upregulated miRNAs; yellow squares represent downregulated miRNAs; blue circles represent genes; lines represent association between the miRNA and the gene. The network is for the assessment of regulatory status of miRNAs and genes. The degree (size) of square represents regulatory functionality of miRNA (i.e. bigger degree indicates more functions). Similarly, the degree of the blue circle is consistent with the network linkage (bigger circle indicates greater regulation by miRNAs). miRNA, microRNA; MSC, mesenchymal stem cell.
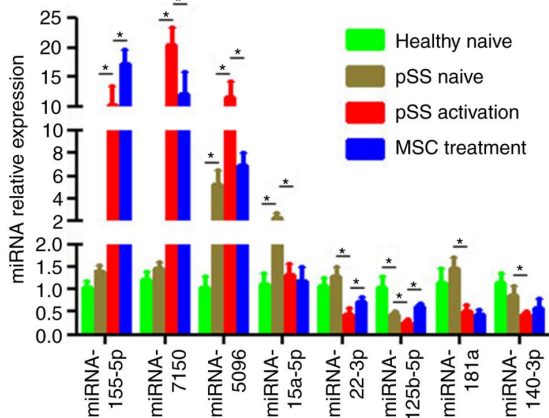

Figure 4. Confirmation of differential expression levels of miRNAs via RT-qPCR. Expression levels of mature miRNA-155-5p, -7150, -5096, -15a-5p, -181a, -125b-5p, -140-3p and -22-3p in CD4+ T cells from healthy and pSS naïve, pSS activation and MSC treatment groups were determined using RT-qPCR. U6 snRNA expression levels were used for normalization. Data are presented as the mean ± SEM of three independent repeats. *P<0.05. miRNA, microRNA; RT-q, reverse transcription-quantitative; pSS, primary Sjögren syndrome; MSC, mesenchymal stem cell.

'axon guidance', 'regulation of actin cytoskeleton' and 'focal adhesion', also showed statistically significant enrichment in

the pSS activation group. 'TCR signaling pathway' remained unchanged in the MSC treatment group (Table SIX). The miRNA-mRNA network revealed that, of miRNAs with high enrichment degree, miRNA-92b-3p, -7704, -762, -7-5p, -4734, -4443, -4267, -22-3p, -17-5p, -150-5p and -106a-5p were upregulated, and miRNA-7150, -6510-5p, -4690-5p, -374b-5p, -30c-5p, -27b-3p, -149-3p, -146a-5p, -142-5p, -1246, -let-7i-5p and -let-7a-5p were downregulated (Fig. 3B; Table SX). miRNA-22-3p and -7150 exhibited a high enrichment degree in the pSS activation group and miRNA-7150 participated in 'TCR signaling pathway'.

qPCR validation in the miRNA microarray. Based on miRNA function classification, certain miRNAs in the miRNA microarray were validated by qPCR. Of the eight aberrant miRNAs in the pSS activation group (Fig. 4), the expression levels of miRNA-155-5p, -7150 and -5096 were upregulated in activated CD4+ T cells while those of miRNA-15a-5p, -181a, -125b-5p, -140-3p and -22-3p were downregulated. Furthermore, upregulation of miRNA-7150 and -5096 and downregulation of miRNA-125b-5p and -22-3p were reversed in the MSC treatment group. Moreover, miRNA-5096 was upregulated in the pSS naïve (vs. healthy naïve) group whereas miRNA-125b-5p was downregulated (Fig. 4). All these results coincided with the microarray results. Although the microarray indicated that
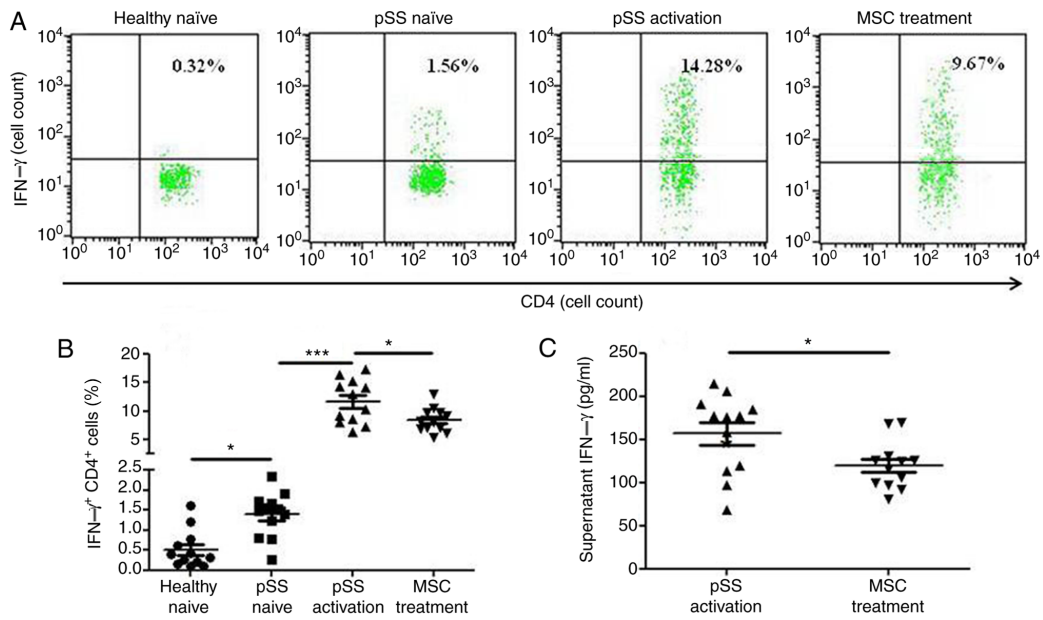
Figure 5. IFN-γ expression levels in CD4⁺ T cells from healthy and pSS naïve, pSS activation and MSC treatment groups. (A) Representative flow cytometric analysis of CD4⁺IFNγ⁺ cells from four groups. (B) Percentage of CD4⁺IFNγ⁺ cells was calculated (n=12). (C) Supernatant IFN-γ secretion from pSS activation and MSC treatment groups was assessed by ELISA (n=12). Data are presented as the mean ± SEM. *P<0.05; ***P<0.001. pSS, primary Sjögren syndrome; MSC, mesenchymal stem cell.
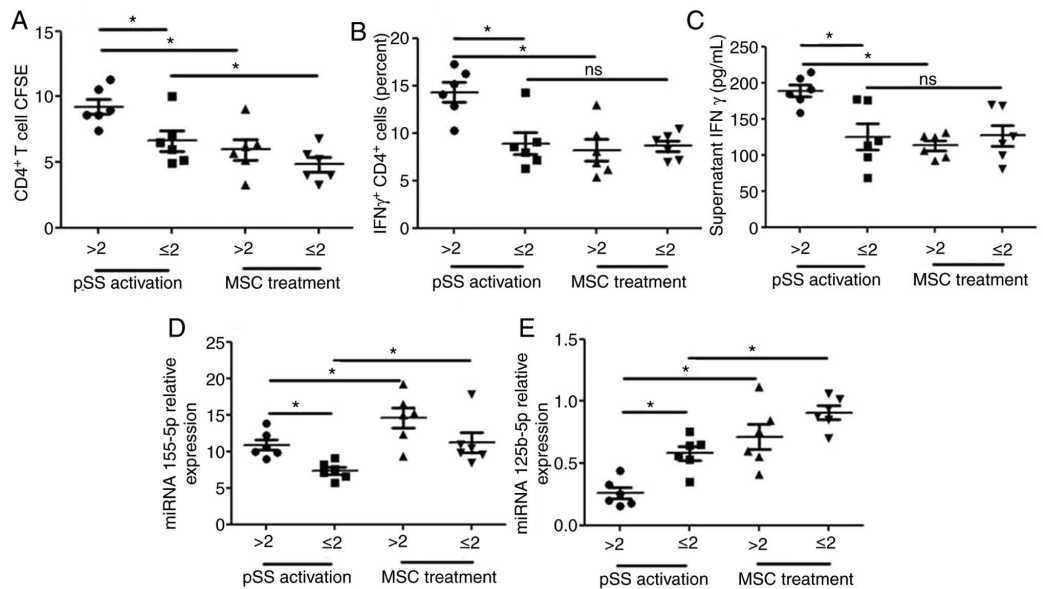

Figure 6. Association between disease activity and miRNA-155-5p/miRNA-125b-5p in activated CD4⁺ T cells from patients with pSS. (A) Proliferation of CD4⁺ T cells, (B) number of CD4⁺ IFN-γ⁺ cells, (C) supernatant IFN-γ secretion and expression levels of (D) miRNA-155-5p and (E) miRNA-125b-5p in CD4⁺ T cells were in patients with inactive (ESSDAI ≤2; n=6) and active disease (ESSDAI >2; n=6) from pSS activation and MSC treatment groups. Data are presented as the mean ± SEM. *P<0.05. miRNA, microRNA; pSS, primary Sjögren syndrome; ESSDAI, EULAR Sjögren's syndrome disease activity index; MSC, mesenchymal stem cell; ns, not significant.

miRNA-155-5p had no 2-fold difference in the pSS activation or MSC treatment groups, qPCR validated a 1.5-fold increase in the MSC treatment group compared with the pSS activation group.

MSCs suppress IFN-γ production in activated CD4⁺ T cells from patients with pSS. CD4⁺ IFN-γ⁺ cells were more prevalent in the pSS activation group than in the pSS naïve group following 72-h co-culture, and MSCs suppressed the levels of

CD4⁺IFN-γ⁺ cells (Fig. 5A and B). Moreover, MSCs decreased the levels of supernatant IFN-γ (Fig. 5C).

Association between disease activity and miRNA-155-5p/miRNA-125b-5p in activated CD4⁺ T cells from patients with pSS. In light of activated CD4⁺ cells in PBMCs from patients with pSS, the present study investigated whether miRNA-155-5p/miRNA-125b-5p in activated CD4⁺ T cells was associated with pSS disease activity. Patients were classified
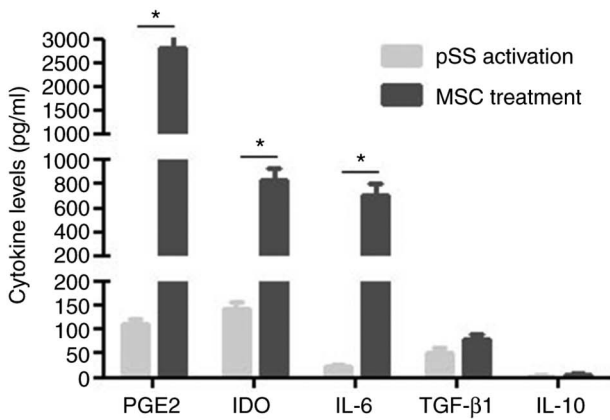
Figure 7. Cytokine secretion profiles of CD4⁺ T cells/MSCs from pSS activation and MSC treatment groups as measured by ELISA. Data are presented as the mean ± SEM of 3 independent experiments. *P<0.05. MSC, mesenchymal stem cells; pSS, primary Sjögren's syndrome; PGE2, prostaglandin E2; IDO, indoleamine 2,3-dioxygenase.

into two groups based on ESSDAI score: ESSDAI ≤2 (n=6) was classified as inactive and ESSDAI >2 (n=6) was considered to be active. The ESSDAI score was associated with alterations in proliferation of CD4⁺ T cells and CD4⁺ IFN-γ⁺ cells, the expression levels of miRNA-125b-5p and miRNA-155 in CD4⁺ T cells and supernatant IFN-γ secretion (Fig. 6). The proliferation of CD4⁺ T and CD4⁺ IFN-γ⁺ cells, the expression levels of miRNA-155 in CD4⁺ T cells and supernatant IFN-γ secretion were notably increased and the expression levels of miRNA-125b-5p in CD4⁺ T cells were significantly decreased in patients with active pSS. MSC treatment reversed these effects on the proliferation of CD4⁺ T cells and CD4⁺ IFN-γ⁺ cells, the expression levels of miRNA-125b-5p in CD4⁺ T cells and supernatant IFN-γ secretion and promoted the expression levels of miRNA-155 in CD4⁺ T cells from patients with active pSS. However, MSC treatment did not reverse the effects on the proliferation of CD4⁺ IFN-γ⁺ cells and supernatant IFN-γ secretion in CD4⁺ T cells from patients with inactive pSS.

MSC-secreted cytokines. MSCs regulate T cells via soluble factors (24). Therefore, potential cytokines for MSC modulation of CD4⁺ T cells were investigated. Activated CD4⁺ T cells alone in culture were revealed to secrete numerous cytokines such as PGE2, indoleamine 2,3-dioxygenase (IDO), IL-6 and -10 and TGF-β1. When MSCs were co-cultured with activated CD4⁺ T cells, the expression levels of PGE2, IDO and IL-6 significantly increased (Fig. 7).

Discussion

To the best of our knowledge, the present study was novel in revealing genome-wide miRNAs in CD4⁺ T cells from patients with pSS following activation and MSC treatment. CD4⁺ T cells relied on signaling pathways that maintain homeostasis between activation and quiescence. Specific miRNAomics detected 55 differential miRNAs between the pSS activation and naïve groups. Teteloshvili et al (25) reported that CD4⁺ T cells of healthy individuals stimulated by CD3/CD28 antibodies exhibited significant activation-induced changes in 12 miRNAs, including upregulation of miRNA-155,

miR-21 and miR-146a. The present miRNA array comprised 38 new miRNAs in the T-lymphocyte function, including upregulated 128 and 131 downregulated GO terms. Moreover, 'TCR signaling pathway' also changed directly, which was targeted by miRNAs such as miRNA-155-5p, -98-5p, -5096, -5787, -181a-5p, -15a-5p, -148b-3p, -140-3p, -7150 and -3609. The present study investigated certain known miRNAs in the T-lymphocyte function. For example, miRNA-155 has been revealed to upregulate the susceptibility of CD4⁺ T cells to natural regulatory T cell-mediated inhibition (26); miRNA-1246 is predominantly expressed in both naïve and memory regulatory T cells (Tregs) (27); and miRNA-15a-5p is displayed in naïve natural Tregs from patients at high risk of type 1 diabetes (28). The loss of miRNA-181a-5p has been demonstrated to alleviate experimental autoimmune encephalomyelitis, attenuate basal TCR signaling in peripheral T cells and decrease their migration from lymph to lesions (29).

MSCs inhibit T cell proliferation and activation and suppress IFN-γ production in CD4⁺ T cells in patients with pSS, but the underlying mechanism remains unclear. In the present study, the effect of MSCs on miRNA expression levels in activated CD4⁺ T cells in patients with pSS was studied; a total of 27 differential miRNAs between the pSS activation and MSC treatment groups was identified. These miRNAs were involved in 117 upregulated and 123 downregulated GO terms. Although the TCR signaling pathway remained unchanged, certain TCR-targeted miRNAs in the pSS activation group, such as miRNA-98-5p, -5096, -7150 or miRNA-155-5p, were reversed or promoted by MSC treatment. Notably, the expression levels of miRNA-155-5p are increased in PBMCs of patients with pSS (7). Upregulated miRNA-155-5p in the pSS activation group was promoted by MSC treatment. Grigoryev et al (30) revealed that knockdown of miRNA-155-5p resulted in significant proliferation of CD4⁺ T cells, confirming that the miRNA-155-5p serves an antiproliferative role during activation. The present findings indicated that MSCs may inhibit mitogenic CD4⁺ T cell proliferation via upregulation of miRNA-155-5p. In addition, although miRNA-125b-5p did not target the TCR signaling pathway directly, both miRNA microarray and qPCR demonstrated that downregulation of miRNA-125b-5p in the pSS naïve group further decreased activation, whereas these effects were reversed by MSC treatment. miRNA-125b-5p was reported to regulate genes associated with T cell differentiation, such as IL2RB, IFNG, PR/SET domain 1 and IL10RA (31); overexpression of miRNA-125b-5p in naïve lymphocytes may inhibit differentiation to effector lymphocytes. miRNA-125b-5p may indirectly participate in TCR activation of CD4⁺ T cells, pSS pathogenesis and MSC treatment for pSS.

The association between ESSDAI and miRNA-155-5p/miRNA-125b-5p in activated CD4⁺ T cells was analyzed. The activated CD4⁺ T cells from patients with active pSS exhibited increased expression of the IFN-γ⁺ phenotype, characterized by the overexpression of IFN-γ and miRNA-155-5p and low expression levels of miRNA-125b-5p. MSC treatment did not change IFN-γ levels in activated CD4⁺ T cells from patients with inactive pSS but did change miRNA-155-5p and miRNA-125b-5p levels in activated CD4⁺ T cells from patients with inactive pSS. miRNA-155-5p

or miRNA-125b-5p may be a more suitable biomarker than IFN-γ when patients with inactive pSS are treated using MSCs. However, further studies are required to confirm this due to the small cohort of patients with pSS.

As for the underlying mechanisms by which MSCs change the miRNome patterns of activated CD4+ T cells, MSCs may affect the alloimmune response via either direct contact or secretory cytokines such as PGE2, TGF-β, IL-10, matrix metalloproteinases and IDO. MSCs suppressed CD4+ T cell proliferation in patients with pSS, which was consistent with the findings of a previous study (3). However, exposure to IFN-γ 24 h before co-culture did not induce greater MSC inhibitory effects on CD4+ T cell proliferation, which was different from the findings of a previous study (22). This may be because the inhibitory effects of exogenous IFN-γ pretreatment were insufficient to overcome the effects of endogenous IFN-γ in activated CD4+ T cells.

MSCs alone or co-cultured with activated CD4+ T cells were revealed to secrete a number of factors, which were reported to affect the miRNA expression levels directly, such as PGE2 (32,33), TGF-β1 (34,35) and IL-10 (36,37), indicating the critical role of these soluble factors of MSCs in miRNA profiles.

The present study had certain limitations. Firstly, the association between miRNA-155/125-5p and pSS pathogenesis was only investigated via co-culturing MSCs with pSS CD4+ T cells. Future studies should use transiently silenced miRNA-155 and/or -125-5p in pSS CD4+ T cells to support the present results. Secondly, miRNA-125b and miRNA-155 changes in systematic RNA array were not significant. This may because be only one RNA array was performed for each group, therefore the miRNA-125b and miRNA-155 was verified by qPCR. Finally, the present study did not demonstrate whether soluble factors or MSCs themselves affect CD4+ activation and cytokine production. This requires confirmation via further experiments, such as RNA interference against Cox-2 in MSCs.

In summary, the present study identified miRNome changes in CD4+ T cells from patients with pSS following activation and MSC treatment, which presented with different miRNA profiles. These miRNAs changed GO terms significantly and were associated with CD4+ T cell proliferation and activation. The TCR signaling pathway induced directly by anti-CD3 and anti-CD28 antibody was affected more profoundly in the pSS activation group than in the MSC treatment group. Upregulation of miRNA-5096 and miRNA-7150 and downregulation of miRNA-22-3p and miRNA-125b-5p in the pSS activation group were reversed by MSC treatment; miRNA-5096 was upregulated and miRNA-125b-5p was downregulated in the pSS naïve group compared with the healthy naïve group, indicating that the two miRNAs may serve a key role in pSS pathogenesis and MSC treatment. Moreover, upregulated miRNA-155-5p was further increased by MSC treatment, implying that MSCs may exhibit immunosuppressant effects in activated CD4+ T cells via the miRNA-155-5p antiproliferative mechanism. The findings demonstrated a key role of miRNAs in CD4+ T cells from patients with pSS following activation and MSC treatment; miRNA-5096, -125b-5p or -155-5p contributed to CD4+ T cell proliferation and activation, which may be crucial for pSS pathogenesis and MSC treatment. Moreover, miRNA-125b-5p and miRNA-155 levels in activated CD4+ T cells from patients with pSS indicated that pSS disease activity could offer a novel biomarker for future MSC management.

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Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JT have made substantial contributions to conception and design of the present study, and acquisition, analysis and interpretation of data. BG was involved in data acquisition and drafting the manuscript. ZL, LZ, JH, JP, SP, MZ and JL contributed to data acquisition. JL also made substantial contributions to conception and design of the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Healthy controls and patients with pSS signed informed consent from Tongji Hospital, Tongji University School of Medicine (Shanghai, China). The participants' rights were protected. All procedures with blood samples and MSCs were confirmed by the Ethics Committee of Tongji Hospital (approval no. K-2012-006; 25 February 2012).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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