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Cyclosporine A Suppresses the Activation of the Th17 Cells in Patients with Primary Sjögren's Syndrome

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ABSTRACT

Primary Sjögren's syndrome (pSS) is a common autoimmune disease involving abnormal Th17 activation. The aim of the current study was to investigate the immunosuppression effect of Cyclosporine A (Cys A), a potent immunosuppressor on the proliferation and activation of T cells, on the activation of Th17 cells.

Blood samples from both inactive and active pSS patients as well as healthy controls were collected and serum and peripheral blood mononuclear cells (PBMCs) were collected and tested for IL-17 and RORγt expression. Subsequently, PBMCs were treated in vitro with Cys A in a series of doses and incubation time and the effect of Cys A on inhibiting Th17 activation was tested by measuring IL-17 and RORγt expression.

IL-17 in both serum and PBMCs as well as RORγt in PBMCs from active pSS patients were significantly elevated on both the mRNA and protein levels comparing to those from both inactive pSS patients and healthy controlCys A in the final concentration of 80ng/ml and the treatment time of 24h showed strong inhibition effect on the expression of IL-17 and RORγt in PBMCs from active pSS patients. However, Cys A in various doses and incubation times did not show much impact on inhibiting IL-17 as well as RORγt expression in PBMCs from healthy donors and inactive pSS patients.

Cys A possesses the capability in immunosuppressing the activation of Th17 cells, suggesting that Cys A may be a potential treatment for pSS and maybe other autoimmune diseases.

Keywords: CyclosporineA; Immunosuppress; Primary Sjögren's syndrome; Th17 cell

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease primarily impairing the body's exocrine glands, specifically the salivary and lacrimal glands.[1-3] By the immune-attack associated with lymphocytic infiltration, the function of the salivary and lacrimal glands are compromised and consequently leads to severe dry eyes and mouth.[4-8] Up to now, there has been a lack of effective cure of pSS and the available treatments are symptomatic.[9,10]

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The pathogenetic mechanisms of pSS have not been fully elucidated. The autoimmune-mediated tissue injury might be driven by immunologically activated glandular epithelial cells which are exposed to autoantigens. Alterations in both innate and adaptive immune responses among people with pSS, such as upregulation of type I interferon-regulated genes, abnormal expression of B-cell-activating factor and activation of the interleukin-23-type T-helper 17 (Th17) cell pathway, have been previously described.[4] Th17 cells are a subset of activated $CD4^+$ T cells that are regulated by the interleukin-6(IL-6)/STAT3/RORγt lineage control and produce IL-17.[11] RORγt, member of the nuclear receptor family of transcription factors, plays an important regulatory role in reducing apoptosis of thymocytes and undifferentiated T cells and promoting their differentiation into pro-inflammatory Th17 cells.[12-14] Recent studies have shown that Th17 cells as well as IL-17produced by Th17 cells play important roles in autoimmune diseases like pSS and the inhibition of activation of Th17 might offer a promising approach for pSS.[15-20]

Cyclosporine A (Cys A), a potent immunosuppressor on the proliferation and activation of T cells, has been widely used in organ transplantation.[21-25] Previous studies have shown that Cys A could reduce lymphocyte infiltration in salivary glands and also reduce the number of activated lymphocytes.[10,26-28] However, whether Cys A could modulate T cell responses in autoimmune diseases like pSS remains unclear. In the current study, we investigated the immunosuppressive effect of Cys A on the activation of Th17 cells in inactive and active pSS patients and healthy donors.

MATERIALS AND METHODS

Ethical Statement

All protocols involving human subjects were reviewed and approved by the Ethical Committee of the Affiliated Hospital of Jiangsu University in accordance with the Declaration of Helsinki.[29] Informed written consents from the participants were obtained in this study.

Patients

50 patients with pSS enrolled in the Affiliated Hospital of Jiangsu University from January 2008 to September 2012 were recruited in this study. Disease diagnoses were established according to the American College of Rheumatology (ACR) criteria.[30] PSS diagnosis was based on the ACR criteria. Case definition required at least 2 of the following 3: 1) Positive serum anti-Sjögren's Syndrome antigen A (SSA) and/or anti- Sjögren's Syndrome antigen B (SSB) or positive rheumatoid factor and antinuclear antibody titer, 2) Ocular staining score≥3, 3) Presence of focal lymphocytic sialadenitis with a focus score ≥ 1 focus/4 mm^2 in labial salivary gland biopsy samples. Exclusion Criteria includes: 1) past head and neck radiation treatment, 2) Hepatitis C infection, 3) acquired immunodeficiency syndrome (AIDS), 4) pre-existing lymphoma, 5) sarcoidosis, 6) graft versus host disease, or 7) current use of anticholinergic drugs. Sjögren's syndrome disease activity index (SSDAI) validated scales was adopted in our study to assess disease activity status. SSDAI evaluates glandular (enlarged parotid glands) and extraglandular features such as fatigue, non-erosive arthritis, skin vasculitis, interstitial lung disease, renal involvement, neurological involvement, myositis, lymphadenopathy/splenomegaly, hematological involvement and fever. The presence of one or more glandular and extraglandular features recognized by SSDAI with the exception of fatigue was defined as clinically active disease.[31] Among 50 patients, 35 patients were in active phase and 15 patients were in inactive phase. Patients in active phase had not received treatment for pSS for 2 years before admission to the hospital. Besides, 50 healthy volunteers were recruited as healthy controls. Characteristics of the pSS patients and healthy donors are summarized in Table 1.

Preparation and Culture of Peripheral Blood Mononuclear Cells (PBMCs)

Blood samples were collected from all pSS patients and healthy donors and (PBMCs were isolated from single buffy coats using a Ficoll-Hypaque density gradient as described previously.[32].

Isolated PBMCs were then cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen) until downstream tests.

RNA Isolation and Real-time PCR Analysis

Total RNA from PBMCs was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Genomic DNA contamination was removed

Table 1. Characteristics of pSS patients and Healthy controls

	Active pSS	Inactive pSS	Healthy controls
Age, mean±SD, years	28±6	30±3	25±7
Sex, male/female	9/26	2/13	25/25

from total RNA with RNase-free DNase treatment (Promega) and cDNA was synthesized using ReverTra Ace® qPCR RT Kit (TOYOBO) following the manufacturer's instructions. The mRNA levels of the target genes (RORγt, IL-17) were detected by quantitative real-time PCR (RT-qPCR) and β-actin was used as an internal control. All PCR reactions were performed on the Rotor-Gene6000 System (Corbett Research). The following primer pairs were used for the RT-qPCR. IL-17: 5'-CAAGACTGAACACCGACT AAG-3', 5'-TCTCCAAAGGAAGCCTGA-3'; ROR γt: 5'-TCTCTCTGCCCTCAGCCTTGCC-3', 5'-CAAG ACTGAACACCGACTAAG-3'; β-actin: 5'-CACGAA ACTACCTTCAACTCC-3', 5'-CATACTCCTGCTT GCTGATC-3'.

Cys A Treatment of PBMCs

For dose assay, Cys A was added into the culture of PBMCs at final concentrations of 20, 40, 60, 80 and 100 ng/ml, respectively. After 24h of treatment, cell culture supernatants and cells were collected.

For treatment time assay, Cys A at a final concentration of 80ng/ml was added into the culture of PBMCs. After 6, 12, 24, 48 and 72 h of treatment, cell culture supernatants and cells were collected.

For both assays, medium and phytohaemagglutinin (PHA, 100ng/ml) were used as negative and positive controls, respectively. IL-17 concentration in culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA). The mRNA and protein levels of IL-17 and RORγt were determined by RT-qPCR and intracellular flow cytometry, respectively.

ELISA

The IL-17 concentrations in serum and PBMC culture supernatants of the pSS patients and healthy donors were detected by ELISA (R&D systems) following the manufacturer's protocols. Data were graphed as fold change of IL-17 concentration in the control group, namely, serum or medium culture from healthy control group.

Intracellular Flow Cytometry

Intracellular flow cytometry was performed as previously described with modifications.[33] In brief, 1×10^6 PBMCs were first stained with anti-CD4-FITC (BD Biosciences) for 20 min at 4°C, and then washed, fixed and permeabilized and stained with anti-IL-17-PE/anti-RORγt-PE (all from BD Biosciences) for another 20 min at 4°C. Background staining was assessed by isotype-matched control antibodies. All cytomeric evaluations were performed on BD FACS Calibur Flow cytometer (Becton Dickinson) and data were analyzed using WinMDI 2.8 software. For gating strategies, cells were first gated on $CD4^+$ (FITC) and side scatter parameters, then IL-17^+(PE) or RORγt^+(PE) cells were gated based on $CD4^+$ cell subsets.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). All statistical analyses were performed using SPSS13.5 statistical analysis software. For comparisons between two groups, paired or unpaired Student's t test was used and $p<0.05$ was considered statistically significant.

RESULTS

The Expressionof IL-17 and RORγt Were Elevated in Serum and PBMCs in Active pSS Patients

As shown in Figure 1A, the serum IL-17 level in active pSS patients was significantly elevated, compared to that in healthy donors (p=0.013). Besides, the IL-17 level in inactive pSS patients also demonstrated moderate elevation, although it did not show statistical difference to that in the healthy donors. mRNA and protein levels of IL-17 and RORγt were subsequently determined in PBMCs from healthy donors and pSS patients. RT-PCR analysis showed that IL-17 and RORγt were significantly increased on the mRNA level in PBMCs of active pSS patients compared to those of healthy controls (p=0.0421 and 0.0450 for IL-17 and RORγt respectively, Figure 1B). The mRNA level of the IL-17 and RORγt, although not statistically significant, slightly increased. The flow

cytometry analysis of RORγt as well as intracellular IL-17 in PBMCs also demonstrated increased expression in active pSS patients compared to the inactive pSS groups (p=0.0330 and 0.0076 for IL-17 and RORγt, respectively, Fig. 1C and D). Of note, the RORγt in PBMCs from inactive pSS was also significantly higher than that from healthy donors (p=0.0425, Figures 1C and D).

Cys A Treatment Inhibits IL-17 and RORγt Expression in PBMCs of pSS Patients

First, the dose effect of Cys A was determined. Among the treatment with Cys A in different final concentrations, IL-17 and RORγt in 80ng/ml Cys A group of active pSS patients, comparing to those in untreated cells, demonstrated sharp decrease on mRNA level. IL-17 of the same treated cells also showed decrease on protein level (p<0.05 for all determinations, Figures 2A and 3). Interestingly, Cys A treatment in all tested doses showed very limited impact on the levels of IL-17 and RORγt in PBMCs from healthy donors and inactive pSS patients. IL-17 and RORγt from these two groups remained at similar levels as those without Cys A treatment.

Next, the incubation time effect of Cys A (final concentration at 80ng/ml) was determined. Within the tested time periods, cells from active pSS patients with 24h of Cys A treatment showed significant reduction in IL-17 and RORγt expression on mRNA level (p<0.05 for all determinations, Fig. 2B). IL-17 and RORγt expression on the protein level also decreased in the same treated cells (Figures 3 and 4). Consistent with the results from the Cys A dose assay, the Cys A in various incubations did not show much impact on inhibiting IL-17 as well as RORγt expression in PBMCs from healthy donors and inactive pSS patients.

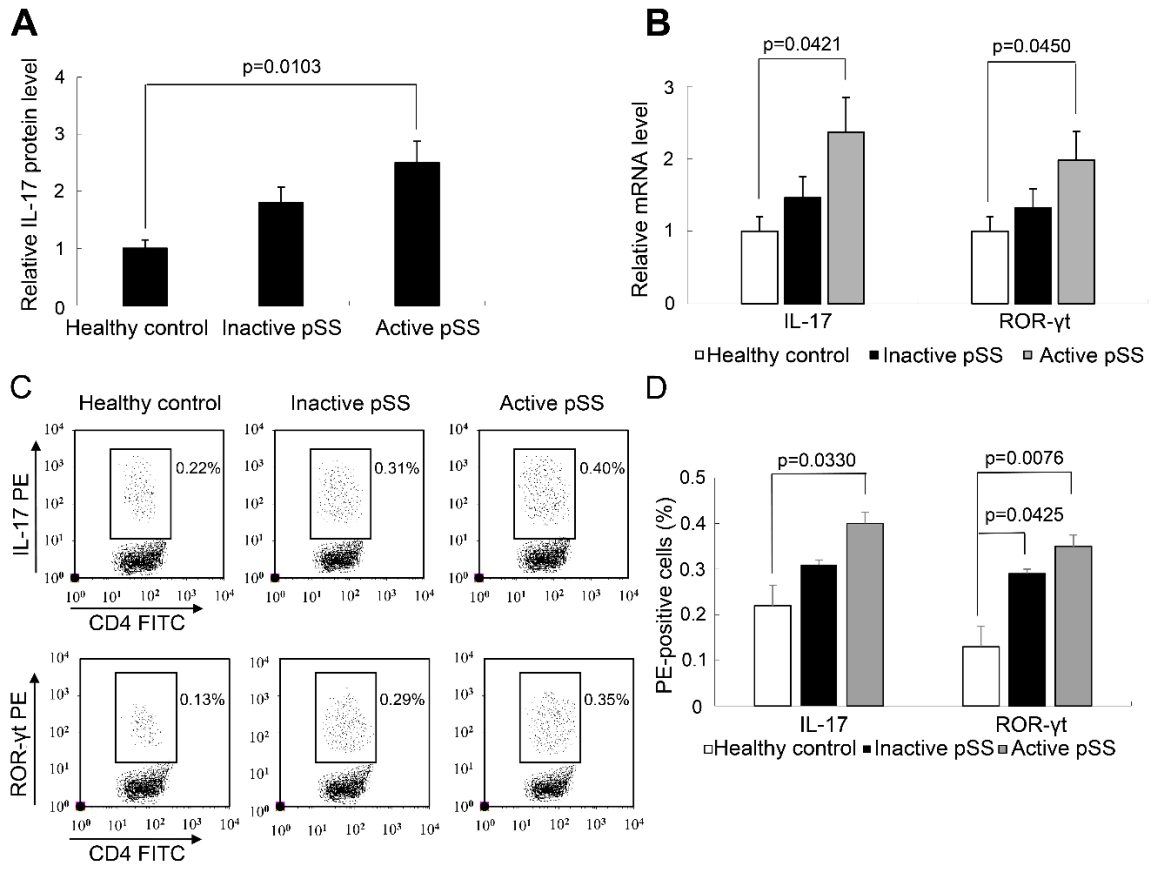

Figure 1. The mRNA and protein levels of IL-17 and ROR-γt in serum and/or PBMCs. (A) IL-17 protein levels in serum were detected by ELISA. (B) RT-PCR analysis of IL-17 and ROR-γt mRNA levels in PBMCs. (C and D) Flow cytometry analysis of IL-17 and ROR-γt expression in $CD4^+$ PBMCs. (C) Representative results of at least three experiments. (D) The percentage of $IL\text{-}17^+$ and $ROR\text{-}\gamma t^+$ cells in $CD4^+$ PBMCs. Data are mean ± SD of at least three experiments.

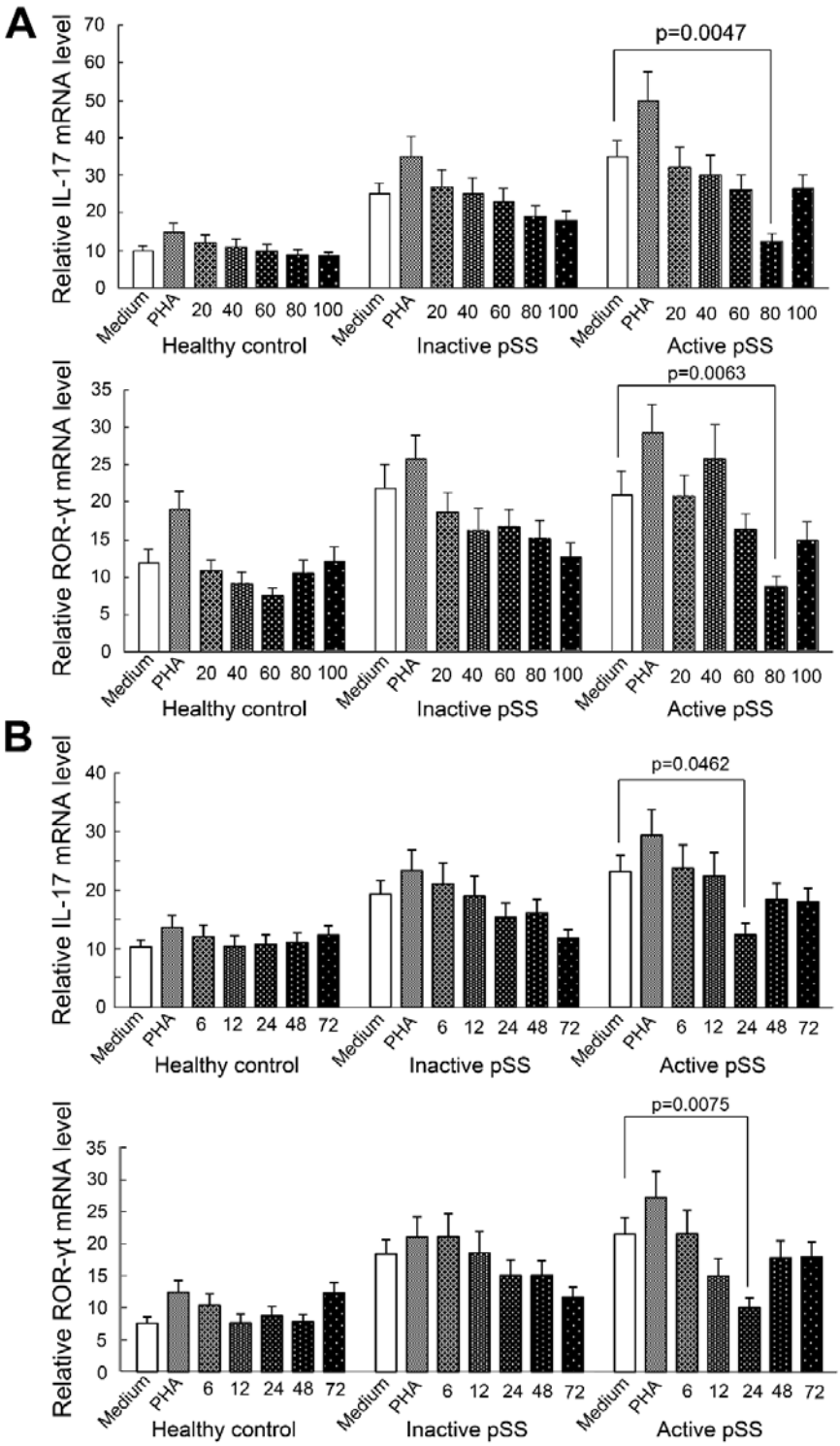

Figure 2. The dose and treatment time effect of Cys A on IL-17 and ROR-γt mRNA expression in PBMCs. (A) The dose effect of Cys A on IL-17 and ROR-γt mRNA expression in PBMCs. Final concentrations of Cys A in 20, 40, 60, 80 and 100 ng/ml were added to treat the PBMCs from three groups and 24h later, cells were collected and mRNA levels were determined by RT-PCR. Data are mean ± SD of three independent experiments. (B) The treatment time effect of Cys A on IL-17 and ROR-γt mRNA expression in PBMCs. Final concentrations of Cys A in 80ng/ml were added to treat the PBMCs from three groups and incubated for 6, 12, 24, 48 and 72h, then cells were collected and mRNA levels were determined by RT-PCR. Data are mean ± SD of three independent experiments.

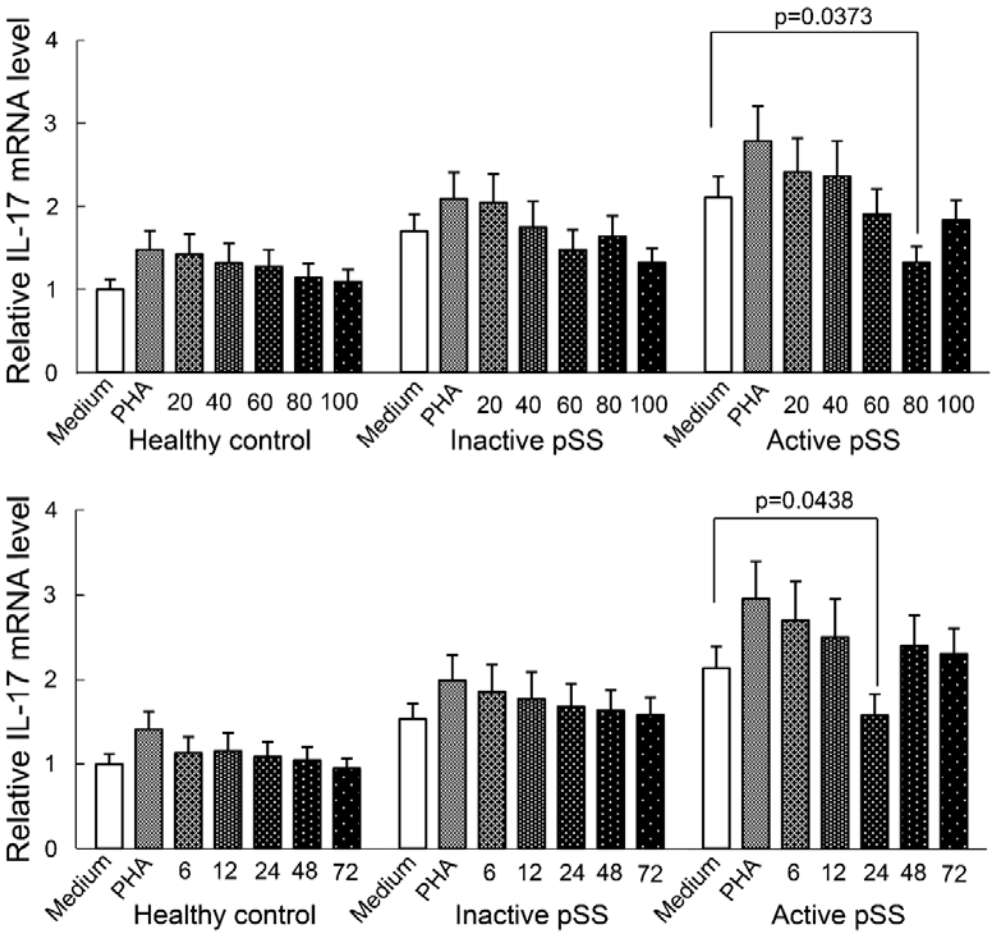

Figure 3. The dose and treatment time effect of Cys A on IL-17 protein expression in PBMCs. (A) The dose effect of Cys A on IL-17 protein expression in PBMCs. Final concentrations of Cys A in 20, 40, 60, 80 and 100 ng/ml were added to treat the PBMCs from three groups and 24h later, cell culture supernatants were collected and IL-17 levels were determined by ELISA. Data are mean ± SD of three independent experiments. (B) The treatment time effect of Cys A on IL-17 protein expression in PBMCs. Final concentrations of Cys A in 80ng/ml were added to treat the PBMCs from three groups and incubated for 6, 12, 24, 48 and 72h, then cell culture supernatants were collected and IL-17 levels were determined by ELISA. Data are mean ± SD of three independent experiments.

DISCUSSION

In the current study, we explored the roles of Cys A in suppression of Th17 cell activation in pSS patients. Our results revealed that IL-17 in both serum and PBMCs as well as RORγt in PBMCs from active pSS patients were significantly elevated on both the mRNA and protein levels. Cys A in the final concentration of 80ng/ml and the treatment time of 24h showed strong inhibitory effects on the expression of IL-17 and RORγt in PBMCs from active pSS patients. Interestingly, Cys A in various doses and incubation times did not show much impact on inhibiting IL-17 as well as RORγt expression in PBMCs from healthy donors and inactive pSS patients. The effect of Cys A on the inhibition of Th17 cell activation in pSS patients but not healthy controls may render it a potential treatment for autoimmune diseases like pSS.

Th17 cells, particularly auto-specific Th17 cells, are associated with autoimmune disease, such as pSS, multiple sclerosis, psoriasis, autoimmune uveitis, juvenile diabetes, rheumatoid arthritis, and Crohn's disease. The Th17 cells would create inflammation and tissue injury in autoimmune disease.[34-36] Consequently,

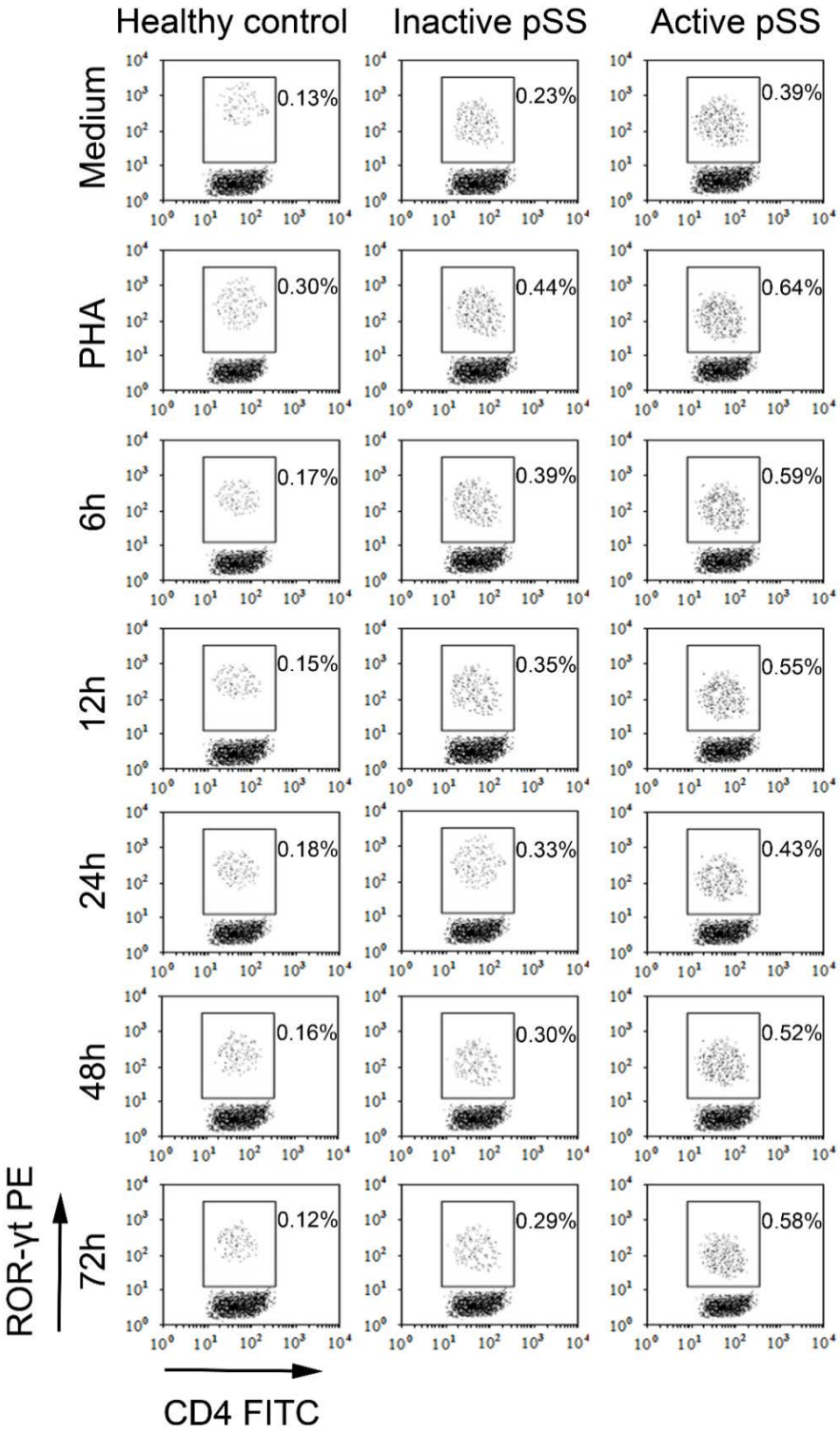

Figure 4. The treatment time effect of Cys A on ROR-γt protein expression in PBMCs. Final concentrations of Cys A in 80ng/ml were added to treat the PBMCs from three groups and incubated for 6, 12, 24, 48 and 72h, then ROR-γt expression in $CD4^+$ PBMCs was determined by Flow cytometry analysis. Representative results of at least three experiments were shown.

the inhibition of activation of Th17 might offer a promising approach for pSS.[18-20] However, since the normal role of Th17 cells is providing anti-microbial immunity at the epithelial/mucosal barriers and a lack of Th17 cells will render the host susceptible to opportunistic infections, the approach of treating autoimmune diseases by suppression of Th17 cell activation needs to within delicately appropriate level, keeping normal Th17 activation intact. In this study, our results showed that Cys A in the final concentration of 80ng/ml and treatment time of 24h is capable of inhibiting IL-17 and RORγt expression in PBMCs from active pSS patients. Whereas Cys A in various doses and incubation times showed little impact on the suppression of IL-17 and RORγt expression in PBMCs from healthy controls and inactive pSS patients, implying that Cys A mainly modulates the unusual high level activation of Th17 cells, but retains the basic Th17 function. Therefore, Cys A might be a treatment for pSS with great potential. Although beyond the scope of the current study, further investigation of Cys A on the treatment of pSS and other autoimmune diseases are warranted.

Cys A has been widely used in organ transplantation as an immunosuppressant drug to prevent rejection. It reduces the activity of the immune system by interfering with the activity and growth of T cells.[21-25,37] Treatment with Cys A may cause a number of serious adverse drug reactions, including nephrotoxicity, neurotoxicity, hypertension, increased risk of squamous cell carcinoma and infections.[38] Therefore, in case Cys A could be investigated on preclinical or clinical tests, such side effects of Cys A should also be monitored.

Taken together, our study found that IL-17 and RORγt are significantly elevated in serum and PBMCs of active pSS patients. The treatment of PBMCs in vitro with Cys A can reduce the expression of IL-17 and RORγt, suggesting immunosuppressive effect of Cys A on the activation of Th17 cells. The effect of Cys A on the inhibition of Th17 cell activation may render it for a potential treatment for pSS and maybe also other autoimmune diseases.

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