

RESEARCH ARTICLE

The role of Th₉ CD4⁺ T cells and IL-9 during primary Sjogren's syndrome

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Abstract

Objective: The objective of this study is to investigate the expression levels of Th₉ CD4⁺ T cells and IL-9 secretion in peripheral blood mononuclear cells of patients with primary Sjogren's syndrome. Further, this study aimed to investigate the role of Th₉ cells in the occurrence and development of pSS.

Methods: A total of 20 pSS patients and 20 healthy people, matched with age and gender, were selected as the experimental and control group, respectively. Flow cytometry and ELISA were used to detect the expression of Th₉ cytokines in peripheral blood mononuclear cells and IL-9 in serum, respectively. These factors were then correlated to other clinical indicators.

Results: The levels of Th₉ CD4⁺ T cells and IL-9 of pSS patients were significantly higher than those of the control group. Th₉ CD4⁺ T cells and IL-9 levels in peripheral blood of pSS patients were negatively correlated with salivary flow rate, while IL-9 level was positively correlated with globulin. The transcription levels of IL-9 and immune-related genes including IL-4, IL-7, IL-17, SMAD3, STAT5 and JAK3 were dramatically increased in serum of pSS patients.

Conclusion: The expression levels of Th₉ in peripheral blood and serum IL-9 of patients with pSS were significantly increased, which were correlated with clinical immunological indexes. Together, these data suggest that Th₉ cells and IL-9 may be involved in the pathogenesis of pSS.

KEYWORDS

ELISA, flow cytometry, IL-9, PBMCs, primary Sjogren's syndrome, pSS, T cells, Th₉

1 | INTRODUCTION

Primary Sjogren's Syndrome (pSS) is a multisystem, autoimmune disease that can progress to lymphoma.¹ Generally, pSS is triggered by genetically dependent environmental factors. During pSS, increased T lymphocyte-induced B lymphocyte hyperfunction and the production of autoantibodies. Then, lymphocytes infiltrate the exocrine gland, leading to the destruction of tissue structure and a loss of functioning.² The clinical manifestations of pSS are varied

and include gland damage, salivary gland enlargement, rash, and recurrent mumps.³ In addition to gland involvement, up to 40% of clinically diagnosed cases of pSS experience deleterious symptoms in the blood, urinary, and lung systems that profoundly affect the quality of life of pSS patients.⁴ Thus, an improved understanding of pSS pathogenesis is urgently needed to implement effective early intervention. In pSS, previous studies have shown that the migration of dendritic cells to the salivary glands induced the production of pro-inflammatory cytokines and promoted the amplification of T

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cells and cellular super mutation of B cells, facilitating salivary gland inflammation.^{5–8} Innate immune-inflammatory responses relevant are activated by microbial components and toxins that promote the secretion of IL-1 and IL-18 which induce the inflammatory hyperactivity observed during pSS.⁹

The dysregulation of cytokines secreted by different cell subsets is the main cause of pSS.^{10–13} Multiple T cell subsets have been found to induce glandular inflammation in pSS. Th17 cells are the main subset of lymphocytes that infiltrate the salivary gland. The main effector cytokine secreted by Th17 cells, IL-17, is an early initiator of T cell-induced inflammatory responses that promotes the release of proinflammatory cytokines and inflammatory response associated with pSS.¹⁴ Additionally, IL-17 can enhance abnormal autoimmune responses by promoting the survival of B cells and T cells and protecting T cells from apoptosis, which together, contribute to glandular damage in pSS patients.^{15–17} As a novel CD4⁺ T cell subset, T helper 9 cells (Th9) have been shown to secrete high levels of the IL-9, which initiates early T-cell-induced inflammatory responses and may be involved in the occurrence and development of various autoimmune diseases.^{18,19} Further, Th9 cells have been shown to aggravate inflammatory responses in the pathogenesis of allergic rhinitis, vitiligo, and various cancers by secreting IL-9.^{20–22} However, the roles and mechanisms of Th9 cells during pSS pathogenesis are still unknown.

In this study, the proportion of Th9 cells in peripheral blood mononuclear cells (PBMCs) and the level of serum IL-9 in patients with pSS were detected. Meanwhile, the transcription levels of IL-9 and other immune-related genes were conspicuously observed using RT-qPCR. These parameters when then correlated with additional immunological indicators of pSS patients. The data presented here provide key insight to explore new therapeutic targets for pSS.

2 | MATERIALS AND METHODS

2.1 | Samples collection and preparation

Twenty female patients with initial pSS who were admitted to the Affiliated Hospital of Qinghai University from January 20 to December 20 and met the international classification (diagnostic) criteria of pSS in 2002 were selected. The age was 47.65 ± 10.90 years, excluding infection, tumor and other autoimmune diseases. Twenty healthy female physical examination subjects matched in age and gender during the same period were selected as the control group, with an age of 43.35 ± 6.00 years. There was no significant difference in age between the two groups ($t = 1.546$, $p = 0.133$).

2.2 | Isolation and culture of peripheral blood mononuclear cells

Three ml of fasting elbow venous blood was collected in the morning and heparin was anticoagulated. Peripheral blood mononuclear cells (PBMCs) were separated by lymphocyte separation solution. PBMCs

was suspended in RPMI-1640 medium and inoculated into 24-well plates with 1×10^6 in each well 9/ml, cell culture with PMA (final concentration of 50 ng/ml) (Britain, Abcam company), ion toxin (750 ng/ml) source leaves biological technology co., LTD. (Shanghai) and BFA (10 μ g/ml) (the United States—Vitrogen In company), and then the 24 hole plate cells transferred to 37°C, 5% CO₂ incubator. In training after 4 h, the cells transferred to the sterile centrifuge tube, 1000 RPM, centrifugal 5 min. Discard supernatant, cell precipitation was washed twice with PBS, and PBS was resuspended for backup use.

2.3 | Expression level of PBMCs Th9 was detected by flow cytometry

Mouse anti-human CD4 (BB515) (BD Horizon) and IL-9 (PE) antibodies (BD Pharmingen) were added into cell suspensions, respectively, and incubated for 30 min away from light. After being washed twice with PBS, 1 ml PBS was re-suspended, and the machine was used for detection within 1 h. Using homotype antibody as control, CD4⁺ IL-9⁺ Are Th9 cells.

2.4 | Serum IL-9 levels were determined by ELISA

3 ml of fasting elbow venous blood was extracted from the subjects in the morning, and the serum was routinely separated. Elisa kit (purchased from Wuhan Boshide Biological Co., Ltd.) was used to detect the level of IL-9 in the serum according to the instructions of the kit.

2.5 | RNA extraction and RT-qPCR analysis

Total RNA was extracted from serum using TRIzol Reagent (Invitrogen) according to the manufacturer's recommended protocols. The concentration of RNA was measured by a DS-11 Spectrophotometer (DeNovix, DE, USA). One microgram of total RNA was reverse transcribed into first-strand cDNA with oligo dT primers using the EasyScript All-in-One First-Strand cDNA Synthesis SuperMix for RT-qPCR (TransGen Biotech) following the manufacturer's instructions, and cDNA templates were stored at -20°C until use. RT-qPCR was performed using the same protocols as previously described. The RT-qPCR protocol consisted of an initial heat activation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 40 s. Three biological replicates were performed for each treatment, and each biological replicate consisted of three technical replicates. The relative expression of genes was analyzed using $2^{-\Delta\Delta\text{CT}}$ method, and ACT was used as internal reference.

2.6 | Statistical methods

SPSS25.0 statistical software was used for data analysis. All data were quantitative data, which were normally distributed by Kolmogorov-Smirnov normality test and expressed as mean \pm standard deviation

($\bar{X} \pm S$). Independent sample *T*-test was used for comparison between the two groups. Correlation analysis was carried out by Pearson method with level $\alpha = 0.05$.

3 | RESULTS

3.1 | Peripheral blood mononuclear cells of pSS patients contain higher Th₉ levels

Th₉ cells can secrete inflammatory factors such as IL-9, which play an important role in the pathogenesis and progression of various autoimmune diseases. To investigate IL-9 variance in the in PBMCs of pSS patients, fasting elbow venous blood of 20 pSS patients was collected. Non-pSS healthy patient blood samples were used as the control. No difference was observed in the age, patient history, and health status between both groups ($t = 1.546$, $p = 0.133$). CD4⁺ T-cells in PBMCs were labeled with BB515, and their differentiation was stimulated with PMA. Meanwhile, CD4⁺ T-cell-derived Th₉ cells were labeled with PE. Flow cytometry was deployed to detect the level of IL-9 in PBMCs of PSS patients. The percentage of Th₉ CD4⁺ T cells among total CD4⁺ T-cells in pSS patients was significantly increased relative to the control group ($p < 0.001$) (Figure 1A). The results showed that the number of Th₉ CD4⁺ T cells in the pSS group (3.83 ± 1.12) was significantly higher relative to the control group (0.34 ± 0.16 ; $t = 13.868$, $p < 0.001$; Table S1; Figure 1A). The saliva flow rate of the pSS group (0.50 ± 0.35) was significantly lower than that of control group (4.25 ± 0.64) ($t = -22.875$, $p < 0.001$; Table 1). Additionally, the globulin level in the pSS group (32.73 ± 5.24) was significantly higher than that in control group (23.00 ± 2.62 ; $t = 7.423$, $p < 0.001$; Table 1). There was no difference in esR, C3, and C4 levels between the two groups (Table 1).

3.2 | Th₉ positive cells were negatively correlated with the saliva flow rate in pSS PBMCs

As the main clinical indicators of pSS, we hypothesized that the involvement of Th₉ positive cells in the pathogenesis of pSS may be

closely correlated with saliva flow rate, esR, globulin, complement C3, and C4 levels. Interestingly, the number of Th₉ positive cells was negatively correlated only with the saliva flow rate ($R = -0.688$, $p = 0.008$; Table 2). No correlation between the number of Th₉ positive cells and ESR, globulin, complement C3, and C4 levels in the pSS group was observed (Table 2). These data indicate that Th₉ positive cells may play a role in the pathogenesis of pSS by affecting the saliva flow rate.

Previous studies have shown that the level of IL-9 is been closely correlated with the pathogenesis of various autoimmune diseases.²⁻⁴ To assess whether there was a relationship between IL-9 and pSS, we detected the serum level of IL-9 in pSS patients via ELISA (Figure 2A; Table S2). The level of IL-9 was significantly higher in pSS group (69.23 ± 28.77 pg/ml) relative to the control group (26.58 ± 12.58 pg/ml) ($p < 0.01$; Figure 2A; Table S2). In agreement with the Th₉ result above, serum IL-9 levels were negatively correlated with saliva flow rate ($r = -0.880$, $p = 0.002$; Table 2). Further qRT-PCR assay verified the upregulated transcription of IL-9 in pSS serum (Figure S2A). Typically, immune-related genes including SMAD3, IRF-1, STAT5 were also dramatically induced to upregulate in the pathogenesis of pSS. Together, these lines of evidence suggest that that Th₉ cells could potentially secrete high levels of IL-9 and contribute to pSS pathogenesis. Conversely, IL-9 was positively correlated with globulin in the pSS patients (Table 2). These data indicate that the secretion of IL-9 by Th₉ cells may further induce the increase of globulin in pSS patients.

3.3 | Variations in IL-9 levels affected various biological processes relevant to pSS pathogenesis

As IL-9 was increased by Th₉ cells of pSS patients (Figure 2A), we hypothesized that Th₉ cells may contribute to pSS pathogenesis through the secretion of IL-9. A series of functional and pathway analyses were used to explain IL-9's potential function in pSS pathogenesis (Figure 2). Firstly, an in-depth IL-9 interaction network was constructed and visualized using Cytoscape (Figure 2B). This network showed that IL-9 activity was associated with various

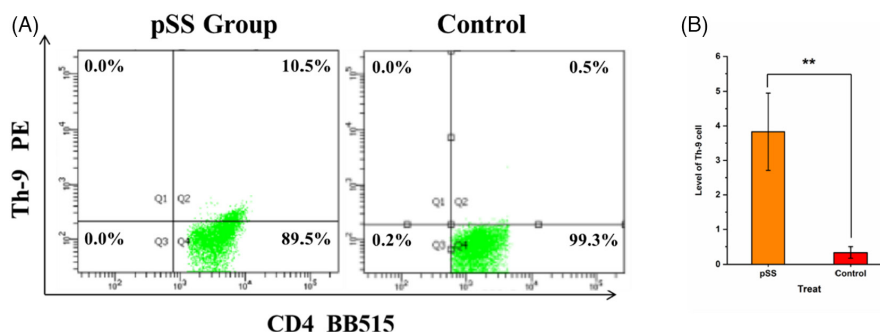


FIGURE 1 Level determination of Th₉ positive cells in peripheral blood of pSS patients. (A) Flow cytometry displayed the low level of Th₉ positive cells in peripheral blood of pSS patients. Representative dot plot showing CD4⁺ and TH-9⁺ cells among peripheral blood mononuclear cells of pSS group and control group. (B) TH-9 level was significantly lower in peripheral blood of pSS patients than that of health persons ($p < 0.01$)

group	PSS group (n = 20)	The control group (n = 20)	T value	p values
Age (years)	47.65 ± 10.90	43.35 ± 6.00	1.546	0.133
Saliva flow rate (ml/15 min)	0.50 ± 0.35	4.25 ± 0.64	22.875	<0.001
Complement C3 (g/L)	1.04 ± 0.16	1.11 ± 0.23	1.19	0.120
Complement C4 (g/L)	0.20 ± 0.04	0.22 ± 0.07	1.414	0.08
Globulin (g/L)	32.73 ± 5.24	23.00 ± 2.62	7.423	<0.001
The ESR (mm/h)	19.40 ± 14.03	14.45 ± 3.75	1.525	0.07

TABLE 1 Comparison of salivary flow rate, ESR, C3, and C4 levels between the two groups

TABLE 2 Correlation analysis on the levels of Th9 positive cells, IL-9 and clinical indicators in the pSS group

	Th9		IL-9	
	r	p	r	p
Th-9/IL9	1	-	1	-
Saliva flow rate	-0.688	0.008	-0.880	0.002
Blood sedimentation	0.178	0.446	0.241	0.162
globulin	-0.104	0.726	0.972	0.001
C3	-0.159	0.637	0.040	0.804
C4	0.125	0.119	0.222	0.451

cytokines, including IL-4, IL-3, IL-13, and IL-7 (Figure 2B). A substantially increased cytokine signature are characteristic of pSS.^{23,24} Additionally, IL-9 was predicted to interact with JAK3, a hub regulator in the JAK-STAT signaling pathway (Figure 2B). Then, we performed qRT-PCR to examine the expression of related genes, and found that the transcription levels of IL-17, IL-4, IL-13 and JAK3 were significantly increased in serum of pSS patients (Figure S2B). Further, gene ontology (GO) enrichment analysis using the genes involved in this complex interaction network (Figure 2C) indicated that these genes mainly functioned as cytokine receptors on the cell membrane. Regarding the cellular component, the external side of the plasma membrane and receptor complex were significantly enriched against these genes (Figure 2C). Further, regarding relevant biological processes, cytokine-mediated signaling pathways, the positive regulation of leukocyte differentiation, lymphocyte activation, and general leukocyte differentiation were identified by GO analysis (Figure 2C). Together, these data indicate the role of IL-9 in the activation of the host immune system. Additionally, results of molecular function reported in the current study support the role of Th₉ cells in pSS pathogenesis through the secretion of various cytokines. Further, receptor activity and cytokine activity were also detected in our analysis (Figure 2C).

KEGG pathway analysis on the genes involved in this network revealed an overrepresentation of various pathways relevant to immune system activation and signaling pathways, including the JAK-STAT signaling pathway, cytokine-cytokine receptor interaction, Th₁ and Th₂ cell differentiation, PI3K-Akt signaling pathway, primary immunodeficiency, and T cell receptor signaling pathways (Figure 2D). Previous studies have validated the observations and shown that

these signaling pathways are involved in pSS pathogenesis and host immune responses.^{25,26} These results suggest that Th₉ cells may contribute to pSS pathogenesis through the secretion of various cytokines that induce the overactivation of host immune responses.

4 | DISCUSSION

pSS is an autoimmune disease that mainly affects exocrine glands such as salivary and lacrimal glands and is characterized by an infiltration of lymphocytes and plasma cells that result in impaired organ function.¹ A variety of etiologies may contribute to the pathogenesis of pSS such as infection, genetics, and endocrine factors. In the current study, we found that an increase in Th₉ cells during pSS was closely associated with clinical indicators of pSS. Furthermore, an increase in Th₉ cells dramatically increased the expression of IL-9, which facilitates inflammatory responses and contributed to the pathogenesis of pSS. GO analysis revealed that IL-9 is a key cytokine important to inflammatory responses and immune-related signaling. Thus, we propose that Th₉ contributes to the pathogenesis of pSS through the induction of IL-9 expression, leading to dramatic inflammatory responses as well as overactivation of immune responses.

Immune disorders are the main contributors to the onset and continuation of the pSS disease. The occurrence of pSS is caused by lymphocytes, including T lymphocytes and B lymphocytes, that infiltrate exocrine glands. T lymphocytes are the main infiltrating cellular subtype with more than 70% being CD4⁺ T cells.²⁷⁻²⁹ The recruitment of these cells to the exocrine glands results in chronic inflammation. Eventually, the tissue structure is destroyed, and the gland function is lost. Biologically, the disruption of the cytokine dynamic balance is the main cause of pSS. CD4⁺ T cells differentiate into different subsets of cells that can secrete a variety of cytokines to elicit several key immune responses.²⁹ At present, the correlation between Th₁, Th₂, Th₁₇, and T_{reg} T cell subgroups and pSS has been confirmed.^{23,30} Th₉ cells are a newly discovered subset of CD4⁺ in recent years. Further, many cytokines are involved in Th₉ cell differentiation. In the current study, we found that Th₉ cells could enhance IL-9 secretion and contribute to inflammation-induced pSS. Inflammatory response is always fine tune by multiple signaling pathways.^{24,25,31} We also observed the upregulations of numerous genes functioned as signaling pathways in serum of pSS patients, such as STAT5, JAK3, STAT6, NF-KB and ILs. Most of these genes were correlated with IL-9 in the PPI network, and involved in

In summary, our study elucidated the role of Th₉ cells and IL-9 in pSS pathogenesis through the induction of inflammatory responses and immune-related signaling pathways. Thus, Th₉ and IL-9 may be novel therapeutic targets for pSS.

AUTHOR CONTRIBUTIONS

Jie Yang involved in methodology, formal analysis, writing the original draft. Juan Su involved in conceptualization, and writing, reviewing and editing. Kexia Chai involved in investigation, writing the original draft, and resources. Huihui Liu involved in software and formal analysis.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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