# Salivary cytokine profiles in primary Sjögren's syndrome differ from those in non-Sjögren sicca in terms of TNF-α levels and Th-1/Th-2 ratios

E.H. Kang[1], Y.J. Lee[1,2], J.Y. Hyon[3], P.Y. Yun[4], Y.W. Song[2]

*[1]Department of Internal Medicine, Seoul National University Bundang Hospital; [2]Department of Internal Medicine and [3]Department of Ophthalmology, Seoul National University College of Medicine; [4]Department of Oral and Maxillofacial Surgery, Seoul National University Bundang Hospital, Seoul, Republic of Korea.*

## Abtract

### Objective

*To compare salivary cytokine profiles in patients with primary Sjögren's syndrome (pSS), non-SS sicca controls, and non-sicca controls, and to investigate whether cytokine levels are correlated with clinical parameters of pSS patients.*

### Methods

*Un-stimulated whole saliva samples were obtained from pSS patients (n=30) classified according to the criteria of the American European Consensus Group. Age- and gender-matched non-SS sicca patients (n=30) and non-sicca subjects (n=25) served as controls. Salivary IFN-γ, TNF-α, IL-1, IL-4, IL-6, IL-10, IL-12p40, and IL-17 levels were measured using a multiplex Luminex® bead-based assay.*

### Results

*pSS patients and non-SS sicca controls had significantly lower salivary flow rates (SFRs) than non-sicca controls, and pSS patients showed a more profound decrease than non-SS sicca controls. In addition, pSS patients and non-SS sicca controls had higher levels of IFN-γ, TNF-α, IL-1, IL-4, IL-10, IL-12p40, and IL-17 in their saliva than non-sicca controls. Salivary TNF-α levels were higher in pSS patients than in non-SS sicca controls. Th-1/Th-2 ratios, represented by INF-γ/IL-4 and TNF-α /IL-4 ratios, were significantly higher in pSS patients than in non-SS sicca controls. SFR was found to be correlated with INF-γ/IL-4 ratio (r=0.411 p=0.024), and focus score to be correlated with TNF-α/IL-4 ratio (r=0.581, p=0.023) in pSS patients.*

### Conclusion

*Th-1, Th-2, and Th17 cytokine levels were found to be elevated in the saliva of pSS patients compared with non-sicca controls. However, considerable overlap was observed between the salivary cytokine levels of pSS patients and of non-SS sicca controls. The features that most differentiated pSS and non-SS sicca were higher TNF-α levels and Th-1/Th-2 ratios. Th-1/Th-2 ratio was also found to be correlated with the clinical parameters of pSS.*

**Key words**

Sjögren's syndrome, sicca, saliva, cytokine

*Eun Ha Kang, MD, PhD*
*Yun Jong Lee, MD, PhD*
*Joon Young Hyon, MD*
*Pil Young Yun, DDS, PhD*
*Yeong Wook Song, MD, PhD*

*Please address correspondence and reprint requests to:*
*Dr Yeong Wook Song,*
*Department of Internal Medicine,*
*Seoul National University Hospital,*
*28 Yongun-dong, Chongno-gu,*
*Seoul, 110-744 Republic of Korea.*
*E-mail: ysong@snu.ac.kr*

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*Competing interests: none declared.*

## Introduction

Sjögren syndrome (SS) is an autoimmune disease characterised by progressive destruction and dysfunction of lacrimal and salivary glands that subsequently manifest the dry eye and dry mouth (sicca symptoms) (1). Lymphoid infiltration in these exocrine glands and the high prevalence of autoantibodies, such as, anti-Ro and anti-La antibodies, support that autoimmune process is involved in the pathogenesis of SS (1). Although the immunopathogenesis of SS remains unclear, evidence suggests that cytokines play a central role; 1) the mRNA expressions of inflammatory cytokines have been consistently shown to be elevated in affected tissues (2-4), 2) cellular studies have demonstrated that cytokine-induced apoptosis of secretory epithelial cells leads to a release of organ specific autoantigens, the generation of autoantibodies, and finally secretory dysfunction (5, 6), and 3) studies on cytokine gene knockout mice have reported that both Th-1 and Th-2 cytokines are critical for the development of SS (7, 8). Although various studies have indicated that Th-1 type cytokines dominate in the minor salivary gland tissues of pSS patients (2-4), results from knock-out mouse systems indicate that both Th-1 and Th-2 cytokines are essential for different stages of disease development (9). In addition, CD4+ Th-17 memory cells have recently been identified within the lymphocytic foci of exocrine glands in SS patients, which indicates the involvement of a complex network of T cell subsets rather than the dominancy of a specific subset of T cells (10, 11).

The sicca symptoms of non-Sjogren's syndrome (non-SS) develop when the secretory functions of lacrimal or salivary glands are significantly affected by any cause other than the autoimmune process, such as, aging, medications, viral infections, and irradiation. Cytokine alterations have been observed in non-SS sicca controls and in non-immune sicca mouse models (12-15). However, few studies have compared the cytokine profiles of SS patients with those of non-SS sicca controls, probably due to the heterogeneity of the latter (12-14). Moreover, Th-17 cytokine levels in these two groups have seldom been compared. In the present study, we undertook to measure Th-1, Th-2, and Th-17 cytokine levels in the whole saliva of SS patients, non-SS sicca controls, and non-sicca controls, and to identify associations between salivary cytokine levels and the clinical parameters of salivary dysfunction.

## Methods

### *Subjects and samples*

Thirty consecutive primary SS (pSS) patients (all female) who met the 2002 revised European classification criteria for SS proposed by the American European Consensus Group (16) were enrolled from January 2003 to January 2008 at Seoul National University Bundang Hospital. Thirty age- and gender-matched subjects who suffered either from dry eye or from dry mouth but did not satisfy the above criteria served as non-SS sicca controls. Twenty-five volunteers who visited our dental clinic due to their chronic gingivitis but were free of other medical illness and did not have any sicca symptoms served as non-sicca controls. Un-stimulated whole mixed saliva samples were collected by spitting for 15 minutes from patients and controls, as previously described (17). Collected saliva volumes were measured using a micropipette after centrifugation at 15000 rpm for 10 minutes at 4°C, and salivary flow rates (SFRs) were calculated. Samples were immediately frozen and stored at -70°C until analysed. This study was approved by the institutional review board of Seoul National University Bundang Hospital (B-0506/021-004) and written informed consent was obtained before study enrollment.

### *Clinical parameters of SS*

Complete blood counts, erythrocyte sedimentation rates (ESR), and the presence of anti-nuclear antibody (ANA), rheumatoid factor (RF), and anti-Ro/La antibodies were determined at the time of saliva collection. The Schirmer test and Rose Bengal staining were performed by an ophthalmologist (JYH). Because we used spitting method rather than cannulation in collecting saliva, oral health status, which could

affect the salivary cytokine profile, was examined using the decayed/missing/filled surface (DMFS) index by a dentist (PYY) (18); the prevalence of dental carries per individual was calculated by measuring the decayed/missing/filled surfaces of teeth. The results of 99mTc-pertechnetate salivary gland scintigraphy were available for 31 of the 60 subjects (19 pSS patients and 12 non-SS sicca controls). Minor salivary gland biopsy was performed in 31 of the 60 (15 pSS patients and 16 non-SS sicca controls). Greenspan's focus score was used to quantify degrees of lymphocytic infiltration (19).

*Cytokine assays*

Salivary IFN-γ, IL-1, IL-4, IL-6, IL-10, IL-12p40, IL-17, and TNF-α levels were analysed using a Luminex 100 system (Luminex Co., Austin, TX, USA) and a LINCOplex® Human Cytokine ImmunoAssay kit (Millipore Co., Billerica, MA, USA), according to the manufacturer's recommendations.

*Statistical analysis*

Salivary cytokine measurements were duplicated and values are presented as mean ± standard deviation (SD). Continuous variables were compared using the Mann-Whitney U-test and categorical variables using the chi-square test or Fisher's exact test. Correlations were evaluated using Spearman's correlation coefficients. *P*-values of less than 0.05 were considered significant, and all statistical calculations were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

## Results

*Clinical characteristics of the study subjects*

The demographic and clinical features of study subjects are summarised in Table I. In general, pSS patients had longer duration of sicca symptoms than non-SS sicca controls ($p$=0.03). Mean SFR (± SD) in the pSS group was severely reduced as compared with those of the control groups ($p$=0.001 versus non-SS sicca controls; $p$<0.001 versus non-sicca controls). Mean SFR was lower in non-SS sicca controls than in non-sicca controls ($p$=0.001).

**Table I.** Demographic and clinical features of study subjects.

| | Primary SS (n = 30) | Non-SS sicca (n = 30) | Non-sicca (n = 25) |
|---|---|---|---|
| Age (years) | 49.9 ± 9.0 | 51.5 ± 10.0 | 49.4 ± 9.5 |
| Gender (F:M) | 30:0 | 30:0 | 25:0 |
| Duration of sicca symptom (years) | 6.1 ± 5.4 | 2.7 ± 2.7 | NA |
| Positive Rose Bengal stain (%) | 5/27 (18.5) | 2/28 (7.1)† | NA |
| Positive Schirmer test (%) | 18/29 (62.1) | 14/30 (46.7)† | NA |
| Salivary flow rate (ml/min) | 0.06 ± 0.05 | 0.15 ± 0.14 | 0.27 ± 0.12 |
| Greenspan's focus score | 2.3 ± 1.3 | 0.63 ± 0.72 | NA |
| Anti-Ro (%) | 25 (83.3) | 1/30 (3.3) | 0 |
| Anti-La (%) | 13 (43.3) | 0 | 0 |
| ANA (%) | 12 (40.0) | 4 (13.3) | NA |
| ESR (mm/hour) | 25.3 ± 19.1 | 9.6 ± 8.3 | NA |
| DMFS index | 42.5 ± 38.2 | 44.5 ± 35.2 | 28.6 ± 18.4‡ |
| Extra-glandular complication | (n=16, %) | | |
| Hypothyroidism | 6 (20) | | |
| Hyperthyroidism | 2 (6.7) | | |
| Raynaud's phenomenon | 4 (13.3) | | |
| Interstitial lung disease | 1 (3.3) | | |
| Primary biliary cirrhosis | 2 (6.7) | | |
| Autoimmune hepatitis | 1 (3.3) | | |
| Renal tubular acidosis | 1 (3.3) | | |
| Ataxic axonopathy | 1 (3.3) | | |

Continuous values are presented as mean ± SD.
ANA: anti-nuclear antibody; DMSF: decayed/missing/filled surface; ESR: erythrocyte sedimentation rates; NA: not applicable.
†$p$>0.05 *vs*. pSS patients; ‡$p$>0.05 versus pSS patients and *vs*. non-SS sicca controls by Mann-Whitney U-test.

Mean focus score ($p$<0.001) and ESR ($p$=0.001) were higher in pSS patients than non-SS sicca controls. The prevalence of ANA ($p$<0.05) and of anti-Ro/La antibodies ($p$<0.0001) was higher in pSS patients than in non-SS sicca controls. DMFS indices were similar in the three groups. Extra-glandular manifestations were observed in 16 pSS patients. There was no case of lymphoproliferative complication (20).

*Salivary cytokine levels*

As shown in Figure 1, all cytokine levels, except for IL-6, were significantly higher in pSS patients and in non-SS sicca controls than in non-sicca controls. IL-6 levels were higher in pSS patients than in non-sicca controls ($p$=0.011), but no difference was observed between non-SS sicca and non-sicca controls ($p$=0.137). Whereas IFN-γ, IL-1, IL-6, and IL-10 levels tended to be higher in pSS patients than non-SS sicca controls, TNF-α levels were significantly higher in pSS patients ($p$=0.002). We then examined if Th-1/Th-2 ratio, as represented by INF-γ/IL-4, IL-12p40/IL-4, or TNF-α /IL-4 ratios, could differentiate pSS patients from non-SS sicca controls. For those whose IL-4 levels were undetectable (n=13 in pSS, n=18 in sicca), the smallest IL-4 value (=0.15 pg/mL) measured among study subjects was used to calculate the ratios. The INF-γ/IL-4 and TNF-α /IL-4 ratios of pSS patients were found to be significantly higher than those of non-SS sicca controls ($p$=0.028 and $p$=0.038, respectively) (Fig. 2). We could not find any particular extra-glandular manifestation associated with cytokine levels that were significantly higher in pSS patients than in non-SS sicca controls.

*Correlation between cytokine levels and the clinical parameters of pSS*

No correlations were found between any salivary cytokine levels and SFRs, focus scores, DMFS index, Schirmer test results, Rose Bengal staining results, ESR, or the presence of ANA and anti-Ro/La antibodies in pSS patients or in non-SS sicca controls. However, in pSS patients, SFR was found to be correlated with INF-γ/IL-4 ratio (r=0.411 $p$=0.024) and focus score to

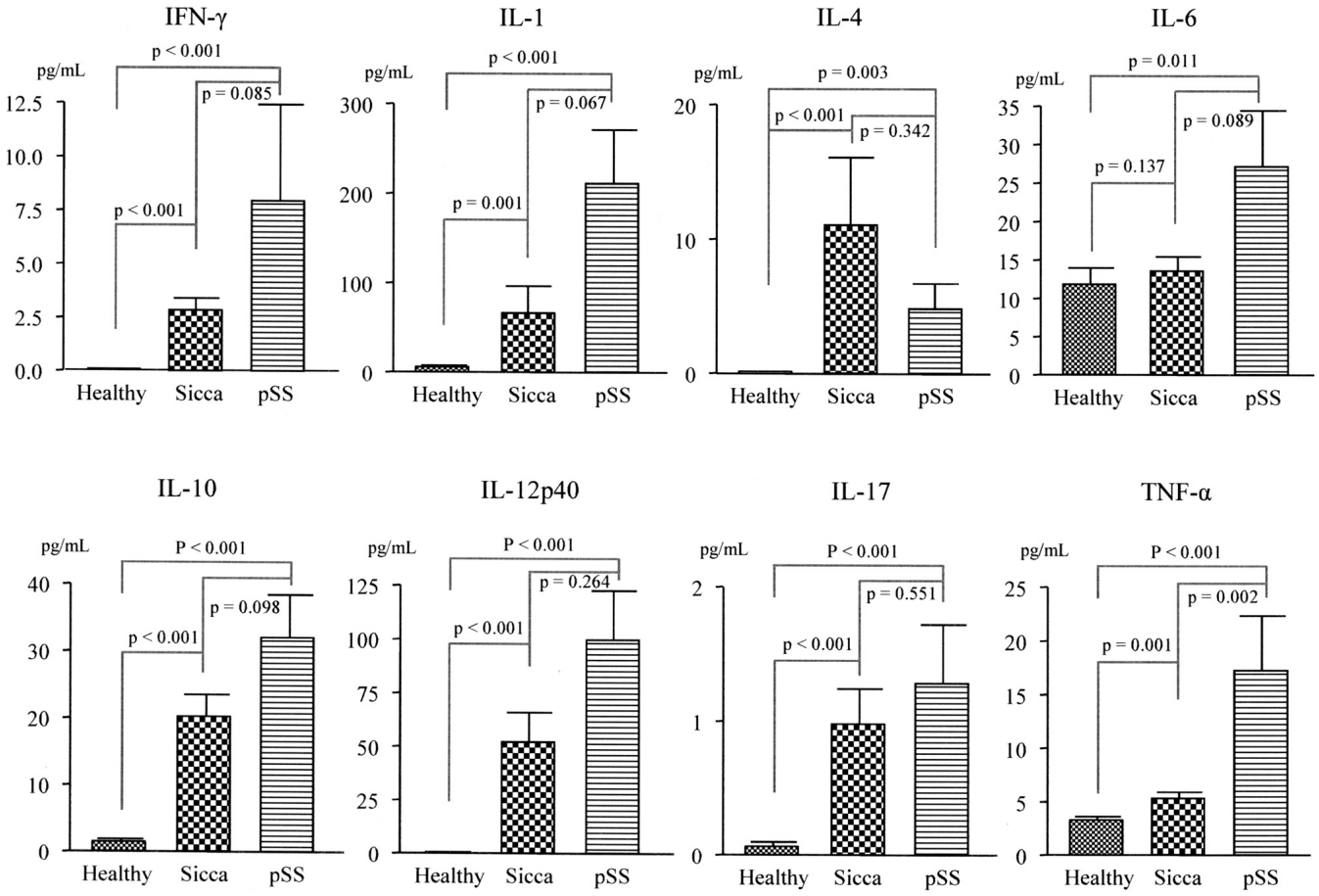


**Fig. 1.** Salivary IFN-γ, IL-1, IL-4, IL-6, IL-10, IL-12p40, IL-17, and TNF-α levels in non-sicca controls (n=25), non-SS sicca controls (n=30), and pSS patients (n=30). Only TNF-α levels were significantly higher in pSS patients than in non-SS sicca controls ($p$=0.002). Bars and error bars indicate means and standard errors of means, respectively.

be correlated with TNF-α/IL-4 ratio (r=0.581, $p$=0.023) (Fig. 3). The focus scores of non-SS sicca controls were found to be correlated with TNF-α/IL-4 ratios (r=0.634, $p$=0.011).

## Discussion

The strengths of the present study are 1) that Th-1, Th-2, and Th-17 cytokine levels and their ratios were comprehensively examined, and 2) that this is one of the few studies to compare pSS patients, non-SS sicca controls, and non-sicca controls. Luminex bead-based technology allowed us to measure the levels of cytokines simultaneously in saliva, even in severely affected patients. Our results showed that almost all Th-1, Th-2, and Th-17 cytokine levels examined were higher in pSS patients and in non-SS sicca controls than in non-sicca controls. TNF-α was the only cytokine found to have significantly higher levels in pSS patients than in non-SS sicca controls. Furthermore, Th-1/Th-2 ratios, including INF-γ/IL-4 and TNF-α /IL-4 ratios, were significantly higher in pSS patients than in non-SS sicca controls.

Our findings that IFN-γ, IL-1, IL-6, IL-10, and TNF-α levels are higher in pSS patients than in non-sicca controls are consistent with previous reports (2-4). In addition, a significant overlap was found between pSS patients and non-SS sicca controls in terms of the levels of almost all cytokines in the present study. This finding is in line with a recent observation by van Woerkom *et al*., who investigated the cytokine profiles of local and peripheral T cells in SS and non-SS sicca patients; a considerable overlap was found to exist in terms of salivary IFN-γ and IL-4 producing T cell frequencies (14). Based on the findings of our study and those of others (12-14), significant elevations in cytokine levels are not limited to pSS but are also a feature of non-SS sicca. However, pSS patients were different from sicca controls in that their immunological activation was found to be skewed to Th-1 rather than Th-2 response, as reflected by a higher Th-1/Th-2 ratio. This finding is worth to notice because Th-1 dominancy reported in the salivary glands of pSS patients has been a feature compared with normal salivary glands in most studies (2-4).

Although Th-1 dominant response has been a prevailing paradigm for the pathogenesis of pSS, several studies reported an over-expression of IL-4 in the salivary glands of pSS patients (4, 21, 22), particularly in association with B cell accumulation (4, 22, 23). Findings obtained using knock-out mouse models indicate that IL-4 is essential for secretory dysfunction in pSS, irrespective of the infiltrating leukocytes (7, 24), and this is because IL-4 modulates secretory function by enhancing

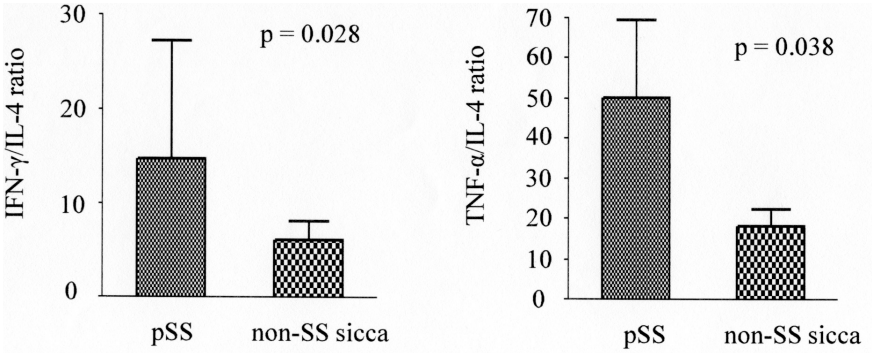


**Fig. 2.** Th-1/Th-2 ratios, represented by INF-γ/IL-4 and TNF-α/IL-4 ratios, were significantly higher in pSS patients than in non-SS sicca controls ($p$=0.028 and $p$=0.038, respectively). Bars and error bars indicate means and standard errors of means, respectively.

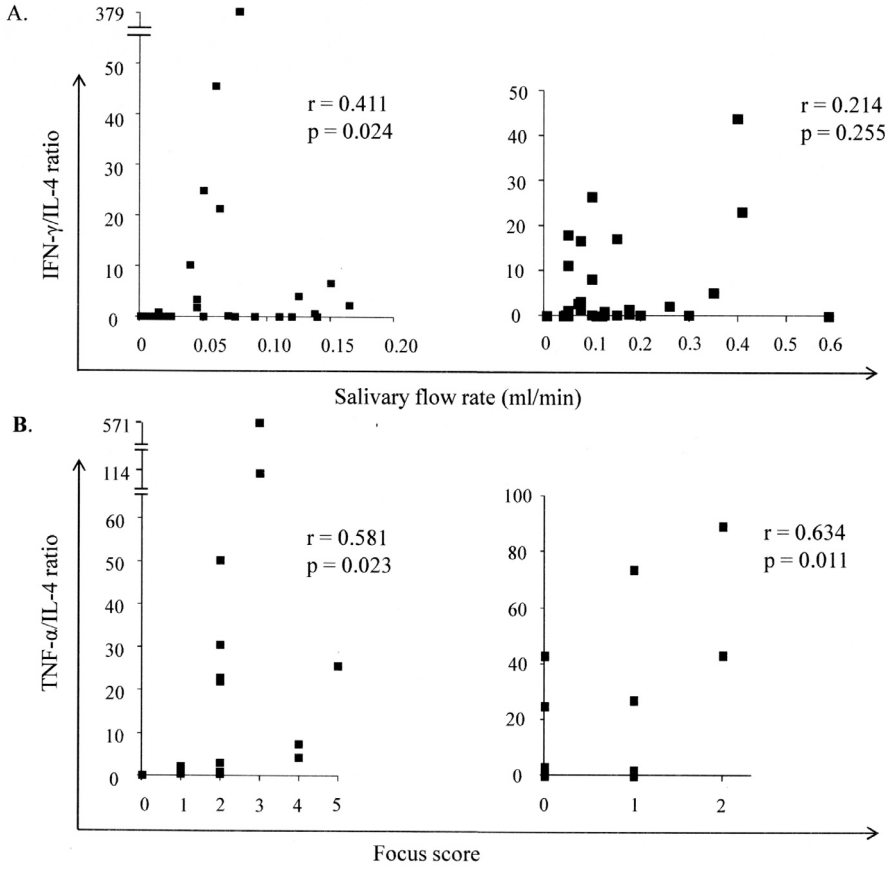


**Fig. 3.** Salivary flow rates were correlated with INF-γ/IL-4 ratios in pSS patients (**A**, left panel), but not in sicca controls (**A**, left panel), and focus scores were correlated with TNF-α/IL-4 ratios in pSS patients (**B**, left panel) and in sicca controls (**B**, right panel).

the production of pathogenic IgG1 autoantibodies, such as, anti-muscarinic receptor antibodies (25, 26). These findings indicate that IL-4 plays an important role during the clinical phase of pSS, although it has little effect on the preclinical exocrine gland pathology (25). Moreover, a recent study showed that Th-1 and Th-2, and even Th-17 cytokines are inducible under the local cytokine milieu in the salivary glands of pSS patients (27). Accordingly, it appears that cytokines of all three classes are activated in pSS but exert their effects at different phases of the disease. In the present study, TNF-α levels and TNF-α/IL-4 ratios were found to be significantly higher in pSS patients than in non-SS sicca controls, and TNF-α/IL-4 ratios were found to be positively correlated with focus scores in pSS patients. These findings support the notion that TNF-α is critical during the pathogenesis of SS. Interestingly, in addition to its role as a potent pro-inflammatory cytokine, TNF-α also has been implicated in the induction of salivary gland cell apoptosis (5, 6, 28) and in the subsequent productions of nuclear autoantigens, such as, Ro, La and α-fodrin (29), and thus, contributes to the productions of pSS-related pathogenic autoantibodies. Moreover, dose-dependent increases in TNF-α converting enzyme expression and subsequent TNF-α production have been demonstrated in human salivary gland epithelial cells treated with anti-Ro/La antibodies (30, 31). These findings suggest that TNF-α plays a key role during the productions of pathogenic autoantibodies and that these autoantibodies are produced by a disease-relevant rather than a non-specific mechanism. However, trials with TNF-α blocking agents in SS have been unsuccessful (32-34). Recent observations have shown that peripheral blood TNF-α and IFN-α levels increase after treatment with TNF-α inhibitor (33, 34), which implies that other pathways negate the benefit of TNF-α blockade. However, it is not known if these alternative pathways are more critical targets than TNF-α, and thus, combined blockade of more than one pathway might be required. Interestingly, TNF-α/IL-4 ratios in non-SS sicca controls also showed a strong positive correlation with focus scores. This might be because some non-SS sicca controls, particularly those with a high focus score and a high TNF-α/IL-4 ratio, were likely to have pre-clinical stage SS or SS not fulfilling the current classification criteria. Longitudinal studies will help clarify if non-SS sicca controls with a high Th-1/Th-2 ratio develop SS, and if the onset of SS in non-SS sicca controls is predicted by an elevated Th-1/Th-2 ratio.

It has been shown that exposure to IFN-γ not only induces glandular cell apoptosis (28) but also directly contributes to secretory dysfunction by disrupting tight junction integrity in salivary gland epithelium (35, 36). The positive correlation found between IFN-γ/IL-4 ratio

and SFR despite the anti-secretory effect of IFN-γ is probably due to IL-4, which is also critical for the induction of autoantibody-mediated secretory dysfunction (7, 24-26).

There are limitations in this study. First, we collected saliva by spitting method rather than cannulation. Therefore, it should be considered that perio-dontal factors could have influenced on the salivary cytokine levels; poor oral health attributed to decreased salivary secretion in pSS patients might have altered oral pathogens and caused falsely higher cytokine levels in this group. However, recent studies have demonstrated that SS patients do not show an increased prevalence of periodontal diseases (37, 38), which is consistent with our results that show similar DMFS scores between three study groups. Moreover, given that the 25 non-sicca controls were those who visited our dental clinic due to their chronic gingivitis, higher cytokine levels in pSS patients do not seem to be a biased result in our study. Second, the correlations found between INF-γ/IL-4 ratios and salivary flow rates and between TNF-α/IL-4 ratios and focus scores in pSS patients were rather weak. Considering the small sample size of this study, interpretation of these results can be challenging.

Taken together, Th-1, Th-2, and Th-17 cytokines were found to be elevated in the saliva of pSS and of non-SS sicca controls compared with non-sicca controls. Furthermore, Th-1 response was more dominant than Th-2 response in pSS patients compared with non-SS sicca controls, and these two groups were differentiated by TNF-α levels and IFN-γ/IL-4 and TNF-α/IL-4 ratios. Finally, SFRs were found to be positively correlated with IFN-γ/IL-4 ratios, and focus scores were found to be positively correlated with TNF-α/IL-4 ratios in pSS patients.

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