T Regulatory Cells Are Markedly Diminished in Diseased Salivary Glands of Patients with Primary Sjögren's Syndrome

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ABSTRACT. ***Objective.*** To investigate the abnormalities of T regulatory cells (Treg) in salivary glands and peripheral blood in patients with primary Sjögren's syndrome (pSS).

Methods. Levels of CD4+CD25+^{high} T cells of the peripheral blood of 52 patients with pSS were measured by flow-cytometric assay. Lower lip salivary gland biopsies were examined by immunohistochemistry, using monoclonal mouse anti-human antibodies [CD25, CD4, CD8, CD68, forkhead transcription factor (Foxp3)] in 30 patients with pSS. Using real-time polymerase chain reaction, Foxp3 messenger RNA expression was assessed in the salivary glands and CD4+ T cells from peripheral blood.

Results. Many inflammatory cells, predominantly CD4+ and CD8+ T cells and macrophages, were found in salivary glands of patients with SS, but CD4+CD25+ Treg numbers and Foxp3 expression were markedly reduced in those biopsy samples. Levels of CD4+CD25+^{high} T cells and Foxp3 expression in peripheral blood of patients with pSS were significantly lower than in healthy controls. However, the inhibitory function of CD4+CD25+ T cells in pSS was unchanged compared to that of controls. Peripheral CD4+CD25+^{high} T cell numbers in pSS did not correlate with Schirmer's test and salivary flow rate, or with the presence or absence of anti-SSA/SSB antibodies and immunoglobulin level.

Conclusion. The remarkable reduction of Treg numbers in salivary glands and reduction of CD4+CD25+^{high} T cells in peripheral blood suggests a possible role for absence of Treg in the pathogenesis of salivary gland destruction in pSS. (First Release Nov 15 2007; J Rheumatol 2007;34:2438–45)

Key Indexing Terms:
PRIMARY SJÖGREN'S SYNDROME T REGULATORY CELLS SALIVARY GLAND

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by lymphocyte infiltration of glandular tissue and autoantibodies against exocrine organs. pSS is a relatively common disease, with a prevalence of 0.33%–0.77% in China[1] and approximately 1% worldwide. It is characterized by destruction of exocrine glands, specifically salivary and lacrimal glands, resulting in oral and ocular dryness due to insufficient secretion[2]. Other glandular tissues that are affected include lungs, skin, hepatobiliary system, and pancreas. Moreover, pSS exhibits extraglandular manifestations such as arthritis, vasculitis, and interstitial lung and renal disease[3]. Some patients with pSS develop lymphoma[4].

The etiology of this autoimmune disease has not been fully elucidated. CD4+CD25+ regulatory T cells (Treg) contribute to maintenance of immune tolerance, and prevent the spontaneous emergence of organ-specific autoimmune diseases[5]. It is now clear that the forkhead transcription factor (Foxp3) acts as a critical regulator in the development and function of Treg. Foxp3 expression has therefore been used as a marker for natural Treg. Animal studies of depletion and replacement of CD4+CD25+ Treg have proven their importance to various autoimmune diseases[6]. In some human autoimmune diseases, numerical or functional abnormalities of CD4+CD25+ T cells and CD4+CD25+^{high} T cells have been reported. For example, numbers of CD4+CD25+ T cells from peripheral blood were significantly reduced in patients with systemic lupus erythe-

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matosus (SLE)[7,8]. Abnormalities of suppressive function but not number of CD4+CD25+ Treg in peripheral blood have been described in patients with multiple sclerosis[9] and type I diabetes[10].

Despite an abundant interest in human peripheral CD4+CD25+ Treg as they may relate to the pathogenesis and perhaps more importantly susceptibility to autoimmune diseases, there are few studies of these cells in affected human organs[11,12]. Our study was designed to examine CD4+CD25+ Treg in diseased salivary glands and peripheral blood of patients with pSS.

MATERIALS AND METHODS

Patients and healthy control population. We investigated 52 consecutive patients (51 women, 1 man) with pSS from the Department of Rheumatology and Immunology, Anhui Provincial Hospital. They were diagnosed according to criteria established by the American-European Consensus Group[13], including positive results for anti-SSA/SSB antibodies or focus score ≥ 1. All patients had primary SS without treatment by any glucocorticoid or immunosuppressive drugs (Table 1). CD4+CD25+ T cells in the peripheral blood of all patients were assessed. Salivary gland biopsies were obtained from 30 patients and 2 healthy controls and 5 patients with viral parotitis. In 22 patients no labial salivary gland biopsy was performed, mainly because of refusal by the patient.

Forty-nine age-matched healthy volunteer adults (mean age 43 ± 14 yrs, 47 women, 2 men) were examined as controls. The research protocol was reviewed and approved by the Hospital Ethics Committee. Informed consent was obtained from all patients and controls.

Immunohistochemistry. Immunohistochemical staining was performed on paraffin sections of salivary glands. Heat-induced antigen retrieval was used on formaldehyde-fixed and paraffin-embedded tissues. After unmasking of antigens, sections were treated with 3.0% H_2O_2 in methanol, and then blocked with 1% bovine serum albumin in phosphate buffered saline. Slides were incubated with biotinylated mouse anti-human CD4 (1/25, NCL-L-CD41F6), CD8 (1/25, NCL-L-CD8-295), and CD68 (1/300, NCL-CD68-KP1) (all Novocastra, Vision BioSystems, Newcastle Upon Tyne, UK). Two antibodies for CD25 staining (1/150 and 1/100; Neomarkers, Labvision, Fremont, CA, USA) and 2 antibodies for Foxp3 (1/100, Abcam, Cambridge, MA, USA; and 1/100, eBioscience, San Diego, CA, USA) were tried in experiments. After testing, anti-CD25 from Labvision and anti-Foxp3 from eBioscience were used to stain cells clearly in salivary glands from patients with pSS and parotitis, and then applied to all slides. After first antibody staining, the slides were incubated with secondary antibody of IgG anti-Fab-HRP (Zymed, San Diego, CA, USA). The sections were reacted with DAB, then dehydrated and mounted. All control samples were incubated with normal mouse IgG instead of the monoclonal antibodies, and showed no nonspecific staining. Chronically inflamed tonsils from humans with non-autoimmune diseases and normal lymph nodes were used as positive controls.

Table 1. Clinical features of patients with pSS (51 women, 1 man).

Clinical Feature	Value
Age, yrs, mean (SD)	46.7 (11.8)
Time from diagnosis, yrs	1.9 ± 2.7
Serology	
Positive anti-SSA, %	70.0
Positive anti-SSB, %	36.9
IgG, g/l, mean ± SD	20.0 ± 6.7
IgA, g/l, mean ± SD	3.6 ± 1.8
IgM, g/l, mean ± SD	2.3 ± 1.6
Function	
Schimer test, mm/5 min, mean ± SD	4.7 ± 5.2
Salivary flow, ml/15 min, mean ± SD	0.26 ± 0.21

Cell suspension. The fresh salivary glands from 7 patients with pSS were cut into smaller segments and mashed gently using a plunger through a 70 μm strainer. Cells were diluted in RPMI-1640 and centrifuged at 1800 rpm for 10 min and resuspended and filtered again through a 40 μm strainer. Cells were collected and stained for flow cytometry.

Antibodies and flow cytometry. Antibodies used for flow cytometry were as follows: phycoerythrin (PE)-conjugated anti-CD4 antibodies (13B8.2); fluorescein isothiocyanate (FITC)–conjugated anti-CD25 (B1.49.9); 3-color reagent kit for T cell subtype (PE-cychrome5–conjugated anti-CD4/PE-conjugated); and the appropriate isotype controls. All antibodies were obtained from Beckman Coulter-Immunotech (Fullerton, CA, USA). Flow cytometry was performed on a Coulter Epics XL flow cytometer using System II software (Beckman Coulter).

All samples were tested as soon as possible after collection (within 2 h). Stained cells were detected rapidly by flow cytometry and analyzed using System II software. The ratio of CD4+CD25+ T cells was calculated by sequential gating on lymphocytes and CD4+ T cells, and expressed as a percentage of CD4+ T cells. The criterion for judging CD4+ CD25+^{high} T cells was a mean fluorescence intensity of CD25 expressed on CD4+ T cells twice that of all CD4+CD25+ T cells.

Proliferation assays. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation from heparinized blood. CD4+CD25+ Treg were isolated from PBMC using CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with a MidiMacs separator unit, according to the manufacturer's instructions. The purity of CD4+CD25+ Treg was > 95%. CD4+CD25+, CD4+CD25– (1.0×10^4), or both cell subpopulations (1:1; 1.0×10^4 each per well) were cultured in 96-well round-bottom plates and were stimulated with anti-CD3/CD28-coupled beads (Dynal Biotech). After 72 h, proliferation reagent WST-1 solution (Roche Diagnostics) was added to each well and incubated for 2 h. The absorbance of samples was measured using filters of 450 nm with 620 nm as the reference wavelength.

Real-time polymerase chain reaction (PCR). CD4+ T cells were enriched from PBMC by negative selection using the CD4+ T cell isolation kit (Miltenyi Biotec). RNA was isolated from salivary glands from 18 patients with pSS and 3 patients with viral parotitis using the RNeasy Mini Kit (Qiagen). The total amount of RNA was reverse-transcribed using Superscript II reverse-transcriptase and oligo (dT) primer in a final volume of 20 μl (Invitrogen, Life Technologies, San Diego, CA, USA). Foxp3 mRNA levels were quantified by real-time PCR using the Rotogene-3000 Real-Time Thermo cycle (Corbett Research, Sydney, Australia). The normalized value for relevant mRNA expression in each sample was calculated as the relative quantity of relevant primers divided by the relative quantity of GAPDH. Sequences of PCR primer pairs were as follows: Foxp3, forward 5'-GCA CAT TCC CAG AGT TCC TC-3' and reverse 5'-ATT GAG TGT CCG CTG CTT CT-3; GAPDH, forward 5'-TGC ACC ACC AAC TGC TTA GC-3' and reverse 5'- GGC ATG GAC TGT GGT CAT GAG-3'. Relative Foxp3 mRNA expression was calculated by dividing the relative quantity of Foxp3 mRNA by the relative quantity of GAPDH in each sample and normalized by setting that of CD4+ T cells and salivary gland tissue from normal control at 100. All samples were expressed as the mean (± SD).

Other assessments. Serum antinuclear antibodies were analyzed by indirect immunofluorescence assay on HEp-2 cell slides. Antibodies against Ro/SSA and La/SSB were detected in serum by immunoblotting. IgG, IgA, and IgM serum levels were measured by immunoturbidimetry assay.

Statistical analyses. Data were processed with the GraphPad Prizm 4.0 software (GraphPad, San, Diego, CA, USA). P value of < 0.05 was considered to be statistically significant.

RESULTS

Reduction of CD4+CD25+ cells in salivary glands of pSS. Almost all salivary gland biopsies of 30 patients with SS exhibited infiltration of inflammatory cells; 12 patients showed scattered or focal infiltration of mononuclear cells without parenchyma destruction, 10 showed focal accumulation with parenchyma destruction, and the other 8 exhibited extensive infiltration of mononuclear cells with severe tissue damage (Figure 1). Salivary glands from 5 patients with viral parotitis showed scattered infiltration of mononuclear cells. There was no infiltration of mononuclear cells or visible injury in control samples.

CD4+ T cells comprised the major population of mononuclear cells in salivary glands. CD8+ and macrophages were also prominent in salivary glands. There were virtually no CD4+, CD25+, or CD8+ T cells and rare macrophages in normal salivary samples.

Only rarely were the infiltrating cells seen to express CD25 in pSS ($p = 0.00006$ vs viral parotitis with Fisher's exact test). The overall frequency of CD25 expression on infiltrating lymphocytes was less than 1% by the use of 2 different clones, IL-2R.1 and IG12, and was much less than that in salivary glands with viral inflammation (about 3%–4% of all infiltrated cells, Figure 2). Similarly, Foxp3 staining with antibody from 2 different clones, hFOXY and pch101 set, was found in only 5 of 30 patients with pSS, but in all patients with parotitis ($p = 0.00078$, pSS vs parotitis with Fisher's exact test). Further, Foxp3-positive cells were fewer (< 1% of all infiltrated cells) in samples of patients with pSS compared to those of patients with parotitis (3%–4% of infiltrated cells, Figure 3).

Foxp3 mRNA expression was detected in only 3 of 18 patients with pSS, but in each of 3 patients with parotitis. In addition, Foxp3 gene expression was less expressed in salivary glands of patients with pSS than that in glands of patients with parotitis (Figure 4B).

The cell suspensions from fresh salivary glands biopsy of 7 patients with pSS were assessed by FACScan. No CD4+CD25+ T cells were detected in the salivary glands of these patients.

Reduced CD4+CD25+^{high} T cells in peripheral blood of pSS.

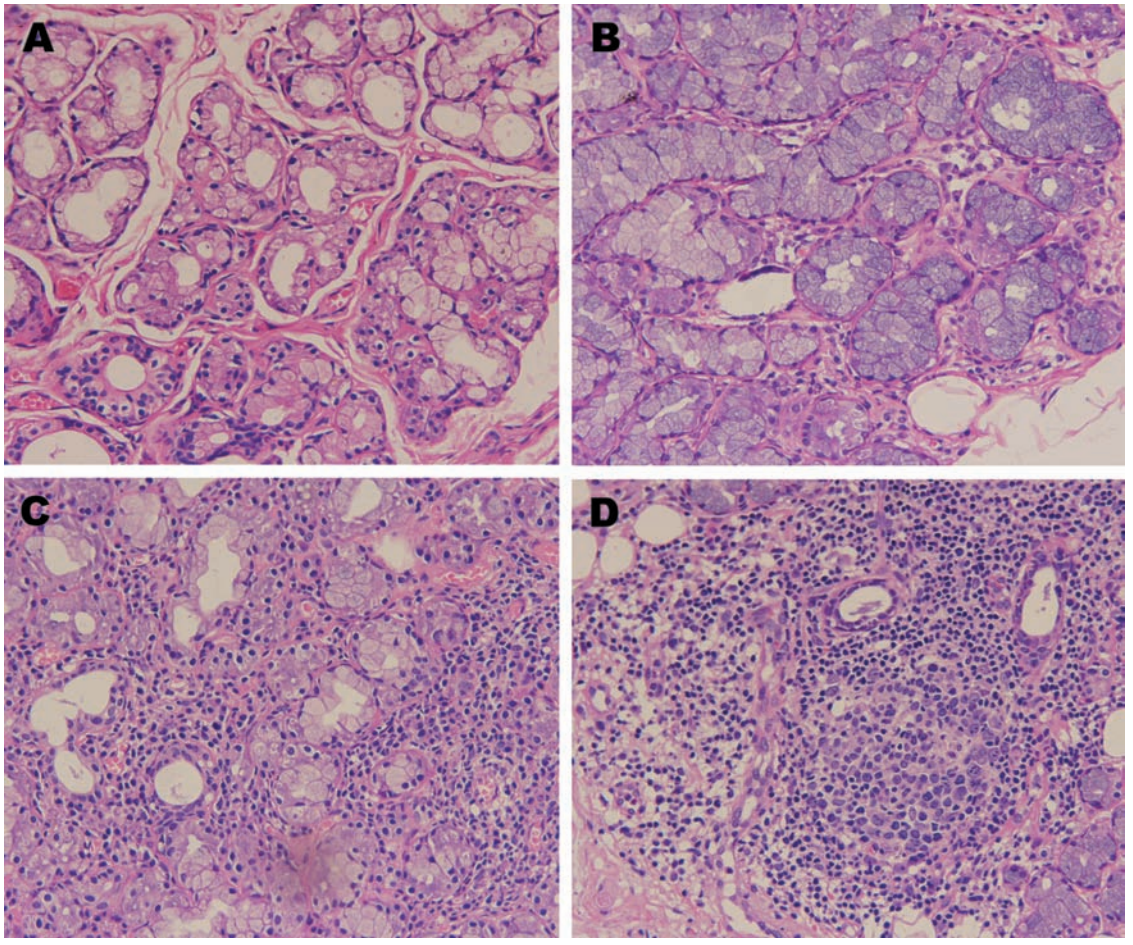

Figure 1. Representative histopathology of salivary glands from patients with pSS (panels B to D) and control (A). B. Scattered infiltration of mononuclear cells without obvious parenchymal destruction. C. Focal infiltration of mononuclear cells in the salivary gland with acinar tissue atrophy and destruction. D. Section showing diffuse infiltration of mononuclear cells in the salivary gland. Staining with H&E (200×).

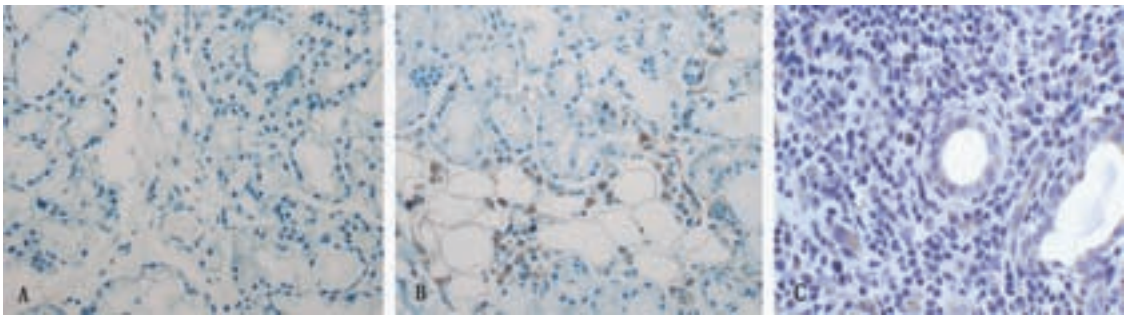

Figure 2. Sections immunostained with the anti-CD25 monoclonal antibody. A. Normal. B. CD25+ T cells in non-autoimmune inflamed salivary glands. C. CD25+ T lymphocytes in salivary glands were detected in only 2 of 30 patients with pSS (400×). Moreover, the number of CD25+ cells was much lower in salivary glands of patients with pSS than in salivary glands from patients with non-autoimmune parotitis.

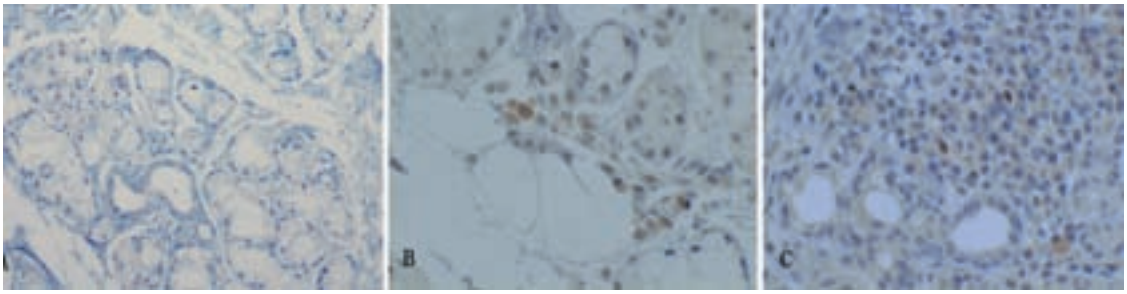

Figure 3. Sections immunostained with the Foxp3 monoclonal antibody. A. Normal. B. Foxp3 expression in the nuclei of T cells in non-autoimmune inflamed salivary glands. C. Foxp3 staining in salivary glands was detected in 5 of 30 patients with pSS (400×). Foxp3-positive cells were markedly reduced in salivary glands of patients with pSS compared to glands from patients with non-autoimmune parotitis.

Forty-nine healthy volunteer adults were examined for CD25 expression on peripheral CD4+ T cells. The percentage of CD4+CD25+^{high} was 1.1% among PBMC (range 0.6 to 3.5%).

In peripheral blood of 52 patients with pSS, the percentage of CD4+CD25+^{high} T cells was significantly reduced (median 0.6%, range 0.001% to 1.8%) compared to controls ($p < 0.001$; Figure 5). Moreover, Foxp3 mRNA expression of peripheral CD4+ T cells was significantly reduced in patients with pSS compared to healthy controls (Figure 4A). However, there was no significant difference in the inhibitory function of peripheral CD4+CD25+ T cells isolated from patients with pSS or healthy controls (Figure 6).

There was no correlation between the levels of peripheral

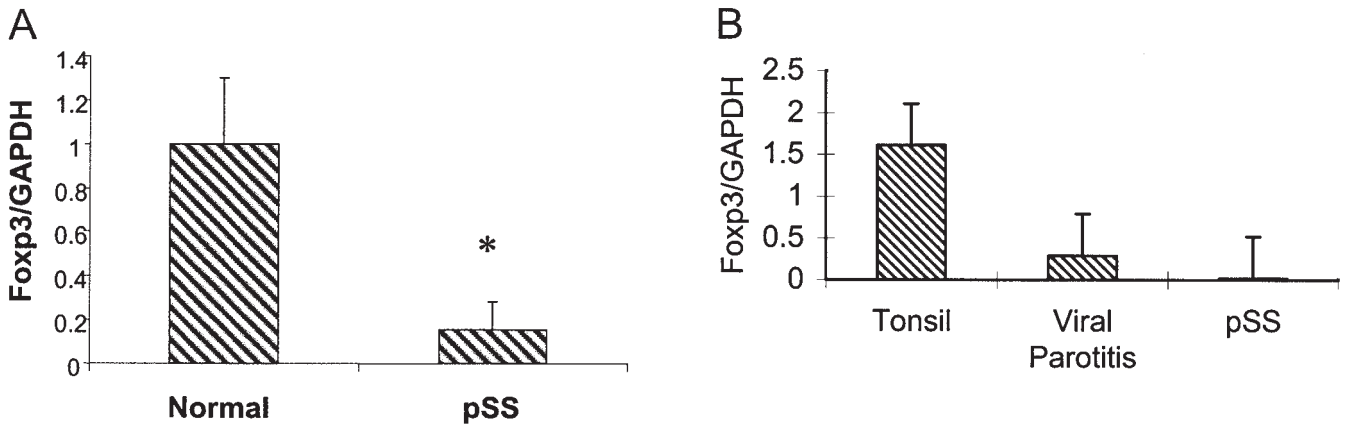

Figure 4. Mean relative Foxp3 mRNA levels assessed by real-time PCR in (A) peripheral CD4+ T cells from 6 healthy controls and 6 pSS patients; and (B) salivary gland tissue from 18 patients with pSS and 3 patients with viral parotitis and 5 tonsils. *$p < 0.05$.

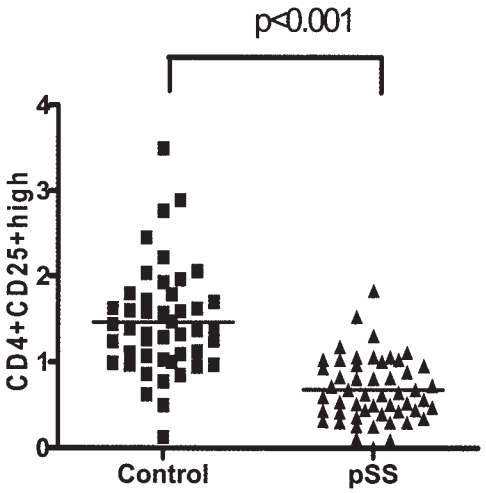

Figure 5. CD4+25+^{high} T cells in peripheral blood were significantly lower in patients with pSS than controls. Values are expressed as percentages of peripheral blood mononuclear cells.

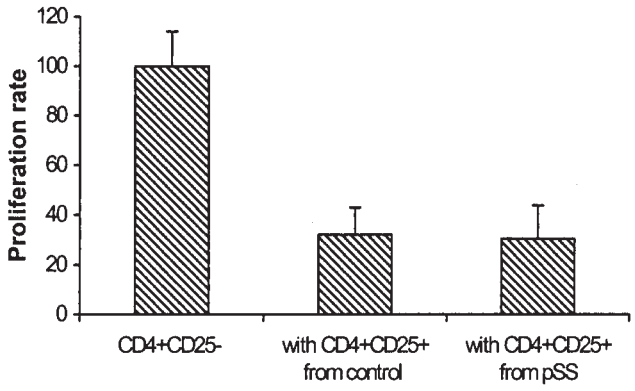

Figure 6. Function of CD4+CD25+ T cells in patients and controls. CD4+CD25– cells were stimulated alone or in the presence of CD4+CD25+ T cells (CD25+:CD25– ratio is 1:1) from 5 healthy controls and 5 patients with pSS. There was no significant difference in the suppressive ability of CD4+CD25+ T cells between controls and patients.

CD4+CD25+^{high} T cells in patients with pSS and presence of serum antibodies against Ro/SSA and La/SSB, serum levels of IgG, IgM and IgA, or exocrine gland function.

DISCUSSION

Our study showed markedly diminished CD4+CD25+ T cells and Foxp3 expression in salivary glands of patients with pSS. Patients with pSS also had a significantly decreased percentage of CD4+CD25+^{high} Treg and reduced Foxp3 expression in their peripheral blood compared to that of healthy controls. However, there was no significant difference in the inhibitory function of CD4+CD25+ T cells of patients with pSS or controls. Moreover, levels of peripheral CD4+CD25+^{high} Treg did not correlate with serological features or functional measures of disease, and so it is difficult to infer a causative role for Treg from the results of peripheral blood testing.

Abundant studies have focused on peripheral levels of CD4+CD25+ and CD4+CD25+^{high} in the patients with autoimmune diseases to explore the role of Treg. However, studies that explore Treg at a target-organ level are very limited[11,12,14-16]. Miyara, *et al* assessed Treg in kidney and lymph node in active SLE, and found that Treg do not accumulate in the sites of inflamed kidney[15]. Similarly, CD4+CD25+^{high} T cells and Foxp3-positive cells were significantly reduced in liver of patients with primary biliary cirrhosis[16]. The most striking finding in our study is rarity of CD25+ cells among CD4 T cells in affected salivary glands of patients with pSS. Previous reports have revealed controversial results on the CD25-positive cell distribution in the affected salivary gland of pSS. Aziz, *et al* reported that although infiltrating mononuclear cells were activated, as evidenced by their expression of HLA-DR and ICAM-1, they did not express CD25, a result confirmed using 2 antibodies[17]. Jonsson, *et al* showed few lymphocytes expressed interleukin 2R[18]. However, Riccieri, *et al*[19] and Coll, *et al*[20,21] reported separately that CD25 was expressed not only on infiltrating lymphocytic cells, but also on epithelial cells in affected salivary glands of pSS. The discrepancies among these reports could relate to specificity and sensitivity of antibodies, the fact that CD25 defines not only regulatory T cells but also activated lymphocytes, and to differences in patient populations. Despite these reported inconsistencies in CD25 expression, it is possible to clarify whether T regulatory cells exist in salivary glands by examining for Foxp3 expression. Foxp3 is not only a key gene in the control and maintenance of Treg function, but also the best marker available to define Treg. In our studies, both immunohistochemisty and real-time PCR analysis confirmed lower Foxp3 expression in salivary gland of pSS compared to that of parotitis, further confirming diminution of Treg.

The reason for diminished Treg in salivary glands of patients with pSS could relate to levels of transforming growth factor-ß (TGF-ß). TGF-ß was strongly expressed in the ductal epithelial cells of normal salivary glands, but was not detectable in the salivary glands of patients with pSS[22,23]. Although one report had contradictory findings regarding ductal expression of TGF-ß, that report still has shown that TGF-ß expression decreased in benign lymphoepithelial lesions in comparison with controls[24]. Emerging evidence has revealed that TGF-ß not only has a role in Treg suppression[25,26], but also in derivation of Treg from naive T cells and prevention of Treg apoptosis[27,28]. Whether remarkably diminished CD4+CD25+ Treg at a target-organ level could contribute to the pathogenesis of pSS is not clear. It is possible that Treg have no role in the pathogenesis, and their diminution could be the results of target destruction, and not the cause of pSS. This possibility is further supported by the evidence that decreased levels of Treg did not correlate with clinical and serological findings. Another explanation is that the diminution in Treg may be involved in the pathogenesis of pSS, since local deficiency of CD4+CD25+ Treg would tip the balance in favor of tissue destruction from CD4+ T cells,

CD8+ T cells, and macrophages. Salivary gland B cells and natural killer (NK) cells may also contribute to the pathogenesis of pSS[29-31]. It has been reported that CD4+CD25+ T cells are able to suppress both B cells and NK cells *in vitro*[32-35]. Whether diminished CD4+CD25+ Treg contributes to the overactivation of these cells in salivary glands remains to be studied further. The importance of local versus peripheral CD4+CD25+ T cells is emphasized by their mechanism of action. CD4+CD25+ Treg have been demonstrated *in vitro* and *in vivo* to exert their suppressive effects in a cell-contact manner[36]. Thus, optimal limitation of inflammation in target organs by Treg requires their presence locally. Interestingly this finding of markedly diminished CD4+CD25+ Treg in the affected organ contrasts with that of rheumatoid arthritis (RA), where CD4+CD25+ T cell enrichment in synovial fluid has been reported[11]. However, in contrast to our study, levels of CD4+CD25+ Treg of synovial fluid were reported, but not those of synovium and other joint tissue[11,12].

In autoimmune diseases, Treg are known to diminish autoreactive responses mediated by CD4+CD25– T cells and may influence the onset and progression of autoimmunity. Decreases in peripheral CD4+CD25+ Treg and/or defective suppressor function have been observed in humans with autoimmune disease[7-9,37-41]. However, no significant changes of peripheral frequency and/or impairment of suppressor function of Treg have been reported in multiple sclerosis or autoimmune polyglandular syndrome type II[9,42]. Moreover, increased levels of peripheral CD4+CD25+^{high} T cells were documented in RA and juvenile idiopathic arthritis[10,12,43].

Discrepancies among reported frequencies of peripheral CD4+CD25+ T cells could relate to the nature of individual autoimmune diseases, but also the method of assessment of levels of CD4+CD25+ T cells. The importance of appropriate age-matched controls has been emphasized[10,44,45], because of age-related differences in the percentage of peripheral blood CD4+CD25+ T cells in controls and patients with autoimmune diseases. In our study, peripheral blood CD4+CD25+ T cells and CD4+CD25+^{high} Treg were significantly reduced in the patients with pSS compared to age-matched healthy controls. The finding in our study is clearly different to that of Gottenberg, *et al*[44], in which the population of Treg defined by phenotypic expression of CD4+ and CD25+ did not appear to differ; conversely, CD4+CD25+^{high} Treg were significantly increased in individuals with pSS compared with healthy controls. A major difference in the studies of Gottenberg, *et al* is that many of the patients with pSS (8/19) were treated with immunosuppressive drugs. Immunosuppressive treatment has been demonstrated to increase levels of peripheral CD4+CD25+ T cells in human autoimmune liver disease[46], SLE[47], and myasthenia gravis[48]. In our studies, no patient with pSS had been treated with immunosuppressive drugs, and so the levels of CD4+CD25+^{high} Treg refer to the naive, untreated state of pSS.

Although peripheral CD4+CD25+^{high} levels were reduced in pSS, their inhibitory function was unchanged in pSS compared to controls. This finding is line with the results from other autoimmune diseases such as SLE and RA[15,49]. A correlation between the number of circulating Treg and clinical measures was not established in our study. The lack of correlation is perhaps not surprising given the overlap of peripheral Treg numbers between healthy controls and pSS. However, deficiency of local Treg could be more important in the pathogenesis of pSS. Anti-Ro/SSA and La/SSB can be detected immunohistochemically in ectopic germinal centers of salivary glands of patients with pSS[30,50]. Lack of local Treg could contribute to increased serum levels of anti-Ro/SSA and La/SSB, because Treg regulate humoral responses by a direct suppression of B cells[50,51]. Similarly, functional hallmarks of pSS, such as xerostomia and xerophthalmia, could result from diminished exocrine gland Treg due to impaired inhibition of destructive infiltrating mononuclear cells. Unlike other autoimmune diseases such as SLE[52] and RA[53], pSS lacks comprehensive criteria to assess disease activity. Therefore, it is also not surprising that in our study there was no correlation between peripheral CD4+CD25+^{high} levels and serological markers or functional indices of disease.

This description of markedly diminished Treg in salivary glands, with decreased numbers of CD4+CD25+^{high} Treg in peripheral blood, is the first to demonstrate target-organ-specific perturbation of Treg numbers in patients with pSS.

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