have been shown to be expressed in a human salivary gland (HSG) cell line, as well as in LSGs from pSS patients in vitro [4]. Additionally, in vitro stimulation of an immortalized human salivary gland cell line, HSG cells, with TLR ligands did not induce Akt phosphorylation but rather the phosphorylation of mitogen-activated protein kinases (MAPKs) [4]. However, no detailed kinetic analyses of apoptotic signals and Akt activation in cultured primary salivary gland epithelial cells (SGECs) of pSS patients have been conducted to date. In our series of studies, apoptotic sensitivity to pro-apoptotic signaling in SGECs differed from that in HSGs [7, 8]. For instance, a significant difference in sensitivity to anti-Fas antibody was observed between these cell types. Although HSGs showed sensitivity to a single stimulation with anti-Fas antibody, cultured SGECs required stimulation with both anti-Fas antibody and phosphoinositide-3-kinase (PI3K) inhibitor to induce apoptosis [7]. Since recent studies have shown that TLRs can induce apoptosis in certain types of cells such as human breast tumor cells [9, 10], it is reasonable to speculate that SGECs and HSGs may respond differently to TLR ligands. Thus, findings obtained thus far with SGECs appear to be more relevant to the clinical setting than those of series using HSGs. In the present study, we investigated TLR-mediated cell death and the expression of relevant anti-apoptotic molecules in the SGECs of pSS patients.

Materials and methods

Patients

This study contained three female patients with pSS (age: 62.7 ± 4.7). Diagnosis of pSS was determined by the revised criteria proposed by the American-European Consensus Group [11, 12]. SGECs obtained from a 59-year-old female who showed sicca symptoms without a diagnosis of pSS were used as the normal control. Labial salivary gland (LSG) biopsies were performed after informed consent was obtained from all participants. The study was conducted in accordance with the human experimental guidelines of our institution.

Antibodies and reagents

Anti-cleaved caspase 3 rabbit monoclonal antibody, phosphorylated-Akt S^{473}, phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase, and phosphorylated p44/42 MAP kinase rabbit polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal goat anti-TLR2, 3, and 4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies including donkey anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) and donkey anti-rabbit IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Hoechst dye 33258 was purchased from Sigma (St. Louis, MO, USA). The selective PI3K inhibitor LY294002 was purchased from Calbiochem (La Jolla, CA, USA). Peptidoglycan (PGN) from Staphylococcus aureus and poly (I:C) were purchased from InvivoGen (San Diego, CA, USA) and lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma (St. Louis, MO, USA).

Culture of primary salivary epithelial cells

The method used for culturing SGECs from pSS patients has been described in our previous reports [7, 8]. Briefly, minor salivary gland tissue was excised and cultured in a defined keratinocyte-SFM culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with hydrocortisone (Sigma) and bovine pituitary extract (Kurabo, Osaka, Japan). For immunofluorescence studies, the SGECs were cultured on 12-mm^2 cover slips that were prospectively coated with a Type I collagen, Cellmatrix (Nitta Gelatin, Inc., Osaka, Japan).

Immunofluorescence

The SGECs on 12-mm^2 cover slips were incubated for 10 min in PBS containing 4 % paraformaldehyde at 4 °C, and the cells were subsequently immersed in methanol at −20 °C for 10 min. After the reaction was blocked in 5 % normal horse serum in PBS, the SGECs were incubated in the primary antibodies for 1 h at room temperature. After the cells were washed three times in PBS, the SGECs were incubated with FITC-labeled and TRITC-labeled secondary antibodies in medium supplemented with Hoechst dye 33258 under dark conditions. After incubation with the secondary antibodies, the SGECs were mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA), and were scanned by confocal microscopy (LSM5, PASCAL; Carl Zeiss, Jena, Germany). Control experiments were performed to confirm the isotype specificity of the secondary antibodies.

Induction of apoptosis

After 12 h of growth-supplement starvation, the primary cultured SGECs were treated with poly (I:C) (final concentration: 25 μg/ml) for 24 h.

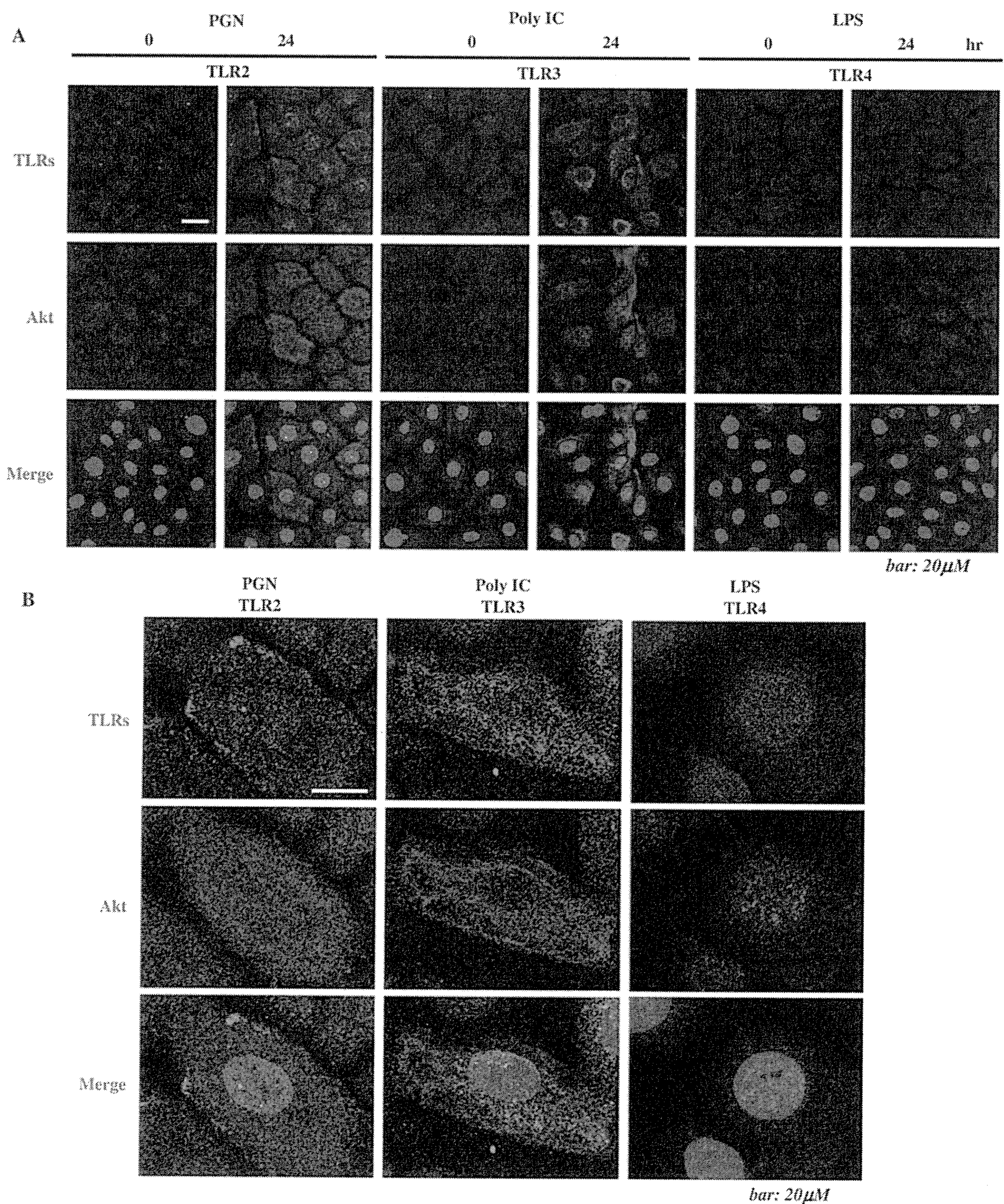

Fig. 1 Expression of TLRs in the presence of their ligands in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 10 μg/ml of PGN, 25 μg/ml of poly (I:C), or 1 μg/ml of LPS for 24 h (low magnification; **a**). The SGECs were double-labeled using goat anti-TLR2, 3, or 4 antibody with FITC-conjugated secondary antibody (*green*) and rabbit anti-phosphorylated Akt antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (*red*). The status of the nucleus was observed by Hoechst staining (*blue*). **b** A higher-magnification view after 24-h stimulation with each ligand. Shown are the representative results of three independent experiments (*bar*, 20 μM)

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining

For the detection of apoptosis, TUNEL staining was employed to demonstrate double-stranded DNA breaks, as shown in our previous study [13]. Later, the SGECs were fixed in 4 % PFA 4 °C for 15 min followed by immersion in PBS with 0.5 % Tween 20 and 0.2 % bovine serum albumin using the MEBSTAIN Apoptosis kit Direct (MBL, Nagoya, Japan). The SGECs were incubated with a 50 µl terminal deoxynucleotidyl transferase (TdT) solution at 37 °C for 1 h. The stained SGECs were captured by confocal microscopy and analyzed by WinROOF software (Mitani Corporation, Fukui, Japan) [14].

Western blot analysis

The method used for Western blot analysis has also been described in our previous reports [8]. Briefly, the SGECs were lysed and the protein concentrations were determined, and identical amounts of protein were subjected to 12.5 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred to a polyvinylidene fluoride filter, blocking for 1 h using 5 % nonfat dried milk in Tris-buffered saline containing 0.1 % Tween 20 was performed, after which the cells were incubated at 4 °C overnight with anti-cleaved caspase 3 rabbit monoclonal antibody, phosphorylated-Akt S^{473}, phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase, and phosphorylated p44/42 MAP kinase rabbit polyclonal antibodies. After incubation with a 1:1,000 dilution of donkey anti-rabbit IgG, coupled with horseradish peroxidase, detection with an enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA) was performed. For statistical analysis, the Student's t test was used ($p < 0.05$; considered as statistically significant).

Results

Expression of TLRs and phosphorylated Akt in primary SGECs with TLR ligand stimulation

We initially examined the expression of three types of TLR in primary cultured SGECs stimulated by TLR ligands (Fig. 1a). Although TLR2 and TLR3 were detected in the cell membrane or cytoplasm in the presence of PGN and poly (I:C), no TLR4 signal was detected (Fig. 1b). Phosphorylated Akt was also detected in the presence of PGN and poly (I:C).

Frequency of nuclear fragmentation under the presence of TLR ligands in primary SGEC from pSS patients and a normal subject

Nuclear fragmentation was detected using Hoechst staining in pSS patients. When 100 cells in three different fields

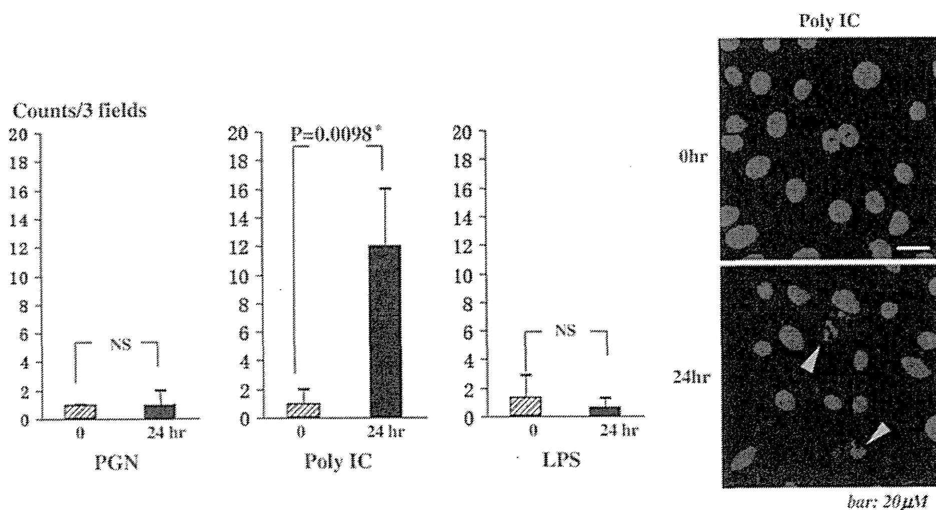

Fig. 2 Nuclear fragmentation induced by poly (I:C) in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 10 µg/ml of PGN, 25 µg/ml of poly (I:C), or 1 µg/ml of LPS for 24 h. Then, to quantify nuclear fragmentation identified by Hoechst staining, 100 cells of interest were counted in three different fields. In the *left panel*, the average number of cell deaths observed among poly (I:C)-stimulated cells was statistically compared with that of PGN or LPS-stimulated cells, as evaluated by unpaired Student's t test ($p < 0.05$; statistically significant). *NS* not significant. Shown are the representative results of two independent experiments. The *right panel* shows representative nuclear fragmentation (*arrowheads*) induced by poly (I:C)

were counted to quantify the fragmented cells, poly (I:C) stimulation induced a statistically significant amount of fragmentation (p value = 0.0098, determined by Student's t test, $p < 0.05$; statistically significant) compared with that induced by PGN or LPS (Fig. 2, left panel). For the normal subject, poly (I:C) stimulation also induced significant nuclear fragmentation (p value = 0.0023). A representative fragmentation in a pSS patient was observed (Fig. 2, right panel).

Detection of poly (I:C)-induced apoptosis by TUNEL assay

TUNEL staining was employed to determine whether the fragmentation determined by Hoechst staining was due to cell death. Twenty-four hours after stimulation with poly (I:C) in SGECs from pSS patients, nuclear fragmentation was detected by bright-field and Hoechst staining. The Hoechst-positive cells were merged, as shown by TUNEL

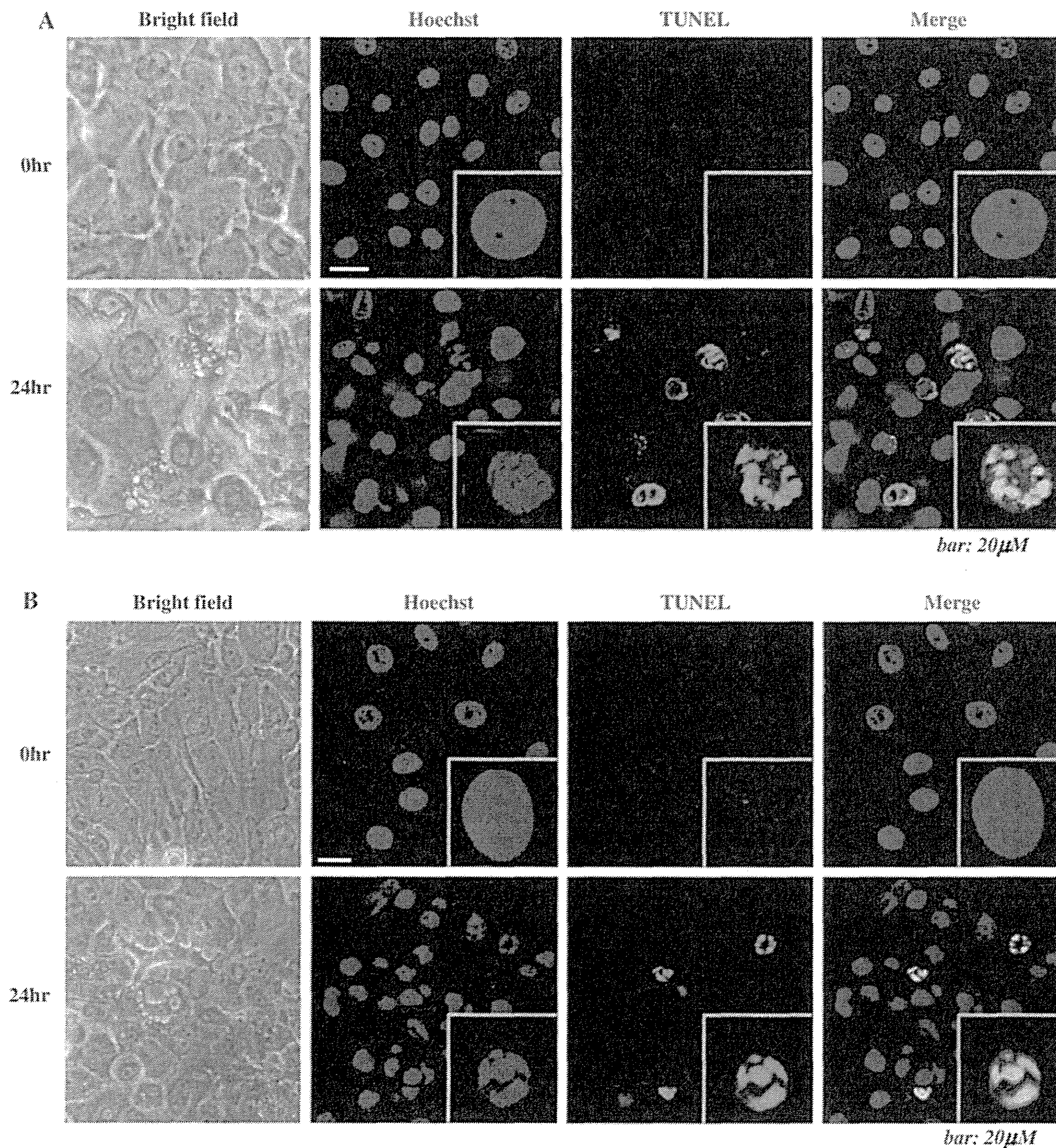

Fig. 3 Detection of double-stranded DNA breakage by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 h. To confirm apoptosis as a double-stranded DNA break at the site of nuclear fragmentation, we employed TUNEL-staining coupled with bright-field view. **a**, **b** The results from a pSS patient and a normal subject, respectively. The inset shows representative staining for each panel. The merged view shows that nuclear fragmentation corresponded to apoptosis (*bar*, 20 μM). Shown in **a** are the representative results of two independent experiments with pSS patients

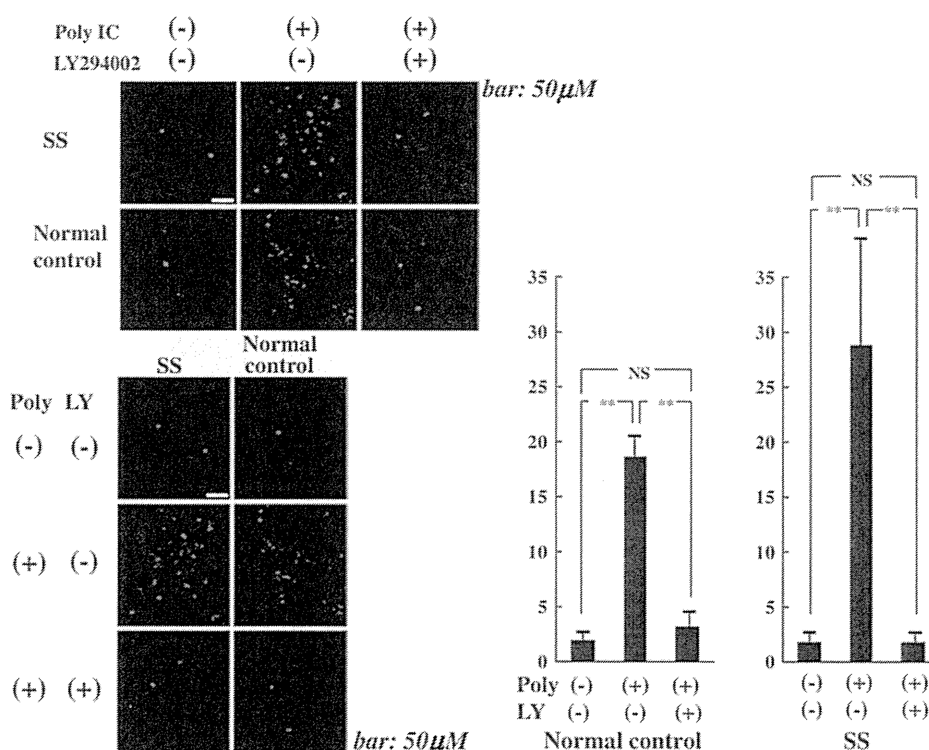

Fig. 4 Quantification of terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)-positive apoptotic cells in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 h. The TUNEL staining image from a pSS patient and a normal subject (*upper panel*) was merged by Hoechst staining (merged view; *bar*, 50 μM). Then, poly (I:C)-induced apoptosis with or without 50 μM of the PI3K inhibitor, LY294002, was detected by TUNEL staining (green) in pSS patients and a normal control, followed by quantification with WinROOF software (pink signal) (*lower panel*). The calculated areas of the captured signals were statistically compared using unpaired Student's t test ($p < 0.05$; statistically significant) (*right panel*). *NS* not significant. Shown are the representative results of two independent experiments

staining (Fig. 3a). Furthermore, poly (I:C)-induced TUNEL-positive cells had merged, as determined by Hoechst staining in the SGECs from one normal subject (Fig. 3b). Poly (I:C)-induced apoptosis detected by TUNEL staining (Fig. 4, upper panel) in pSS patients and the normal control was quantified by converting the TUNEL-positive signal (green) into a pink signal, as observed with an image analyzer (Fig. 4, lower panel); significant acceleration of poly (I:C)-induced apoptosis was seen, as was subsequent inhibition by the addition of a PI3K inhibitor, LY294002, in both groups (Fig. 4, right panel). There was also significant difference of poly (I:C)-induced apoptosis between in both groups ($p < 0.01$).

Akt phosphorylation at the poly (I:C)-induced apoptotic site

To determine whether the phosphorylation of Akt is associated with the poly (I:C)-induced cell death of SGECs, immunostaining of phosphorylated Akt and TLR3 was performed at the site of nuclear fragmentation determined by Hoechst staining. In the SGECs from pSS patients, clear expression of TLR3 and phosphorylated Akt was observed in concert with nuclear fragmentation (Fig. 5, left panel). In the normal subject, poly (I:C)-induced expression of TLR3 and phosphorylated Akt was observed at the site of nuclear fragmentation (Fig. 5, right panel). In the normal subject, poly IC-induced expression of TLR3 and phosphorylated Akt was also observed in cells lacking nuclear fragmentation (Fig. 5, lower panel), which was similar to the co-expression of TLR3 and phosphorylated Akt found in patients with pSS described in Fig. 1b.

Poly (I:C)-induced MAP kinase cleavage of caspase 3 and reversal of effect by PI3K inhibitor

Poly (I:C)-induced expression of MAP kinases including phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase, and phosphorylated p44/42 MAP kinase was performed. Phosphorylation of SAPK/JNK and p44/42 MAP kinase

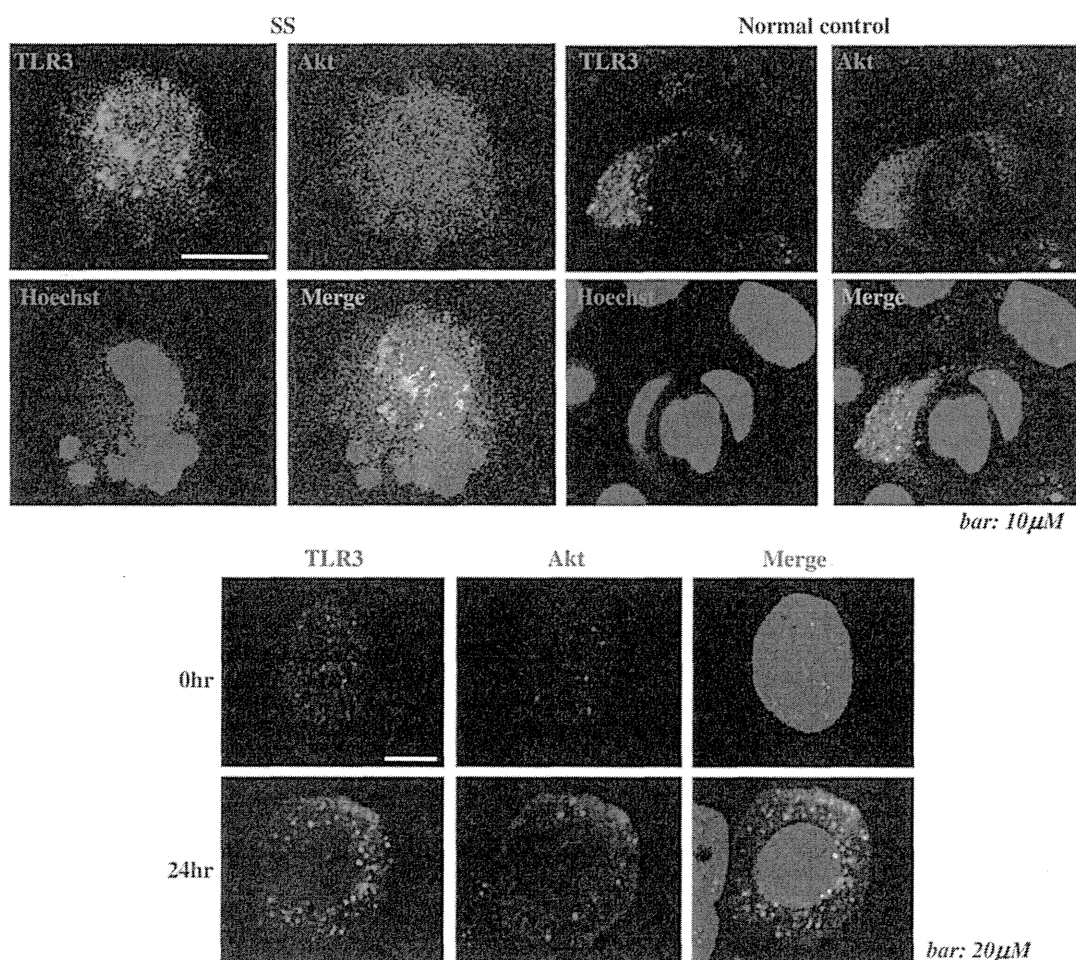

Fig. 5 Co-expression of TLR3 and phosphorylated Akt in apoptotic cells in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 h with or without 50 μM of the PI3K inhibitor LY294002. Nuclear fragmentation detected by Hoechst staining from a pSS patient (*left panel*) and a normal subject (*right panel*) was observed, and the results of Hoechst staining were merged with double-labeled samples using goat anti-TLR3 antibody with FITC-conjugated secondary antibody (*green*), and rabbit anti-phosphorylated Akt antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (*red*). (*Bar* 10 μM). Shown are the representative results of two independent experiments. The *lower panel* shows the expression of TLR3 along with phosphorylated Akt in primary cultured SGECs treated with 25 μg/ml poly (I:C) for 24 h in a normal subject. (*Bar* 20 μM). Shown are the representative results of two independent experiments with pSS patients

was observed, although phosphorylation of p38 was not found. In addition, poly (I:C)-induced signal of phosphorylated SAPK/JNK and p44/42 MAP kinase in pSS was stronger than that in a normal subject. Slight phosphorylation of Akt induced by poly (I:C) was also found. Poly (I:C)-induced cleavage of caspase 3 was examined by Western blot analysis (Fig. 6). Poly (I:C) stimulation clearly revealed cleavage of caspase 3 in the pSS-SGEC lysate by Western blotting, and this result was also obtained in the case of the lysate from the normal subject. Furthermore, cleavage of caspase 3 induced by poly (I:C) was reversed by the addition of LY294002.

Discussion

In this study, TLR-induced apoptosis was clearly observed in the SGECs of pSS patients, as well as in a normal subject. Hsu et al. [15] initially demonstrated that TLRs had the potential to induce MyD88-independent apoptosis in the presence of the protein kinase PKR. Liew et al. [16] reported that TLR2, TLR3, and TLR4 could induce caspase-dependent or -independent apoptosis, in which MyD88-dependent and TIR domain-containing adaptor-inducing IFNβ (TRIF)-dependent pathways were initiated. In addition, Khvalevsky et al. [10] reported that TLR3

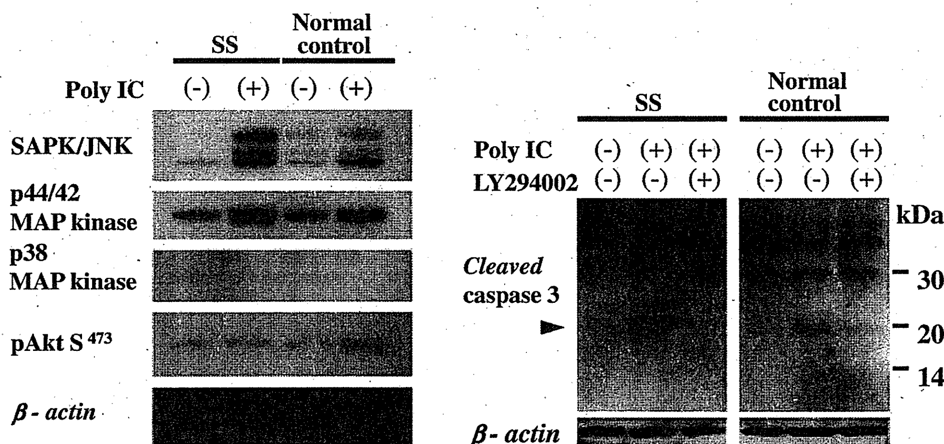

Fig. 6 Detection of poly (I:C)-induced mitogen-activated protein (MAP) kinases and cleavage of caspase 3 in the presence of PI3K inhibitor in primary cultured salivary gland epithelial cells (SGECs). Primary cultured SGECs from a pSS patient or a normal subject were treated with 25 μg/ml of poly (I:C) for 24 h. Then, poly (I:C)-induced expression of Akt and mitogen-activated protein kinases including phosphorylated-stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated-p38 MAP kinase, and phosphorylated-p44/42 MAP kinase was determined by Western blotting (Fig. 6 *left panel*). With or without 50 μM of the PI3K inhibitor LY294002, primary cultured SGECs from a pSS patient or a normal subject were treated with 25 μg/ml of poly (I:C) for 24 h. Then, poly (I:C)-induced cleavage of caspase 3 was determined by Western blotting (Fig. 6 *right panel*). As a control, β-actin was used. Shown are the representative results of two independent experiments with pSS patients

signaling also induced apoptosis in specific cell lines. Usually, in TLR3 signaling, TRIF is recruited after ligand binding, followed by the activation of NF-κB [17] and interferon (IFN) regulatory factor 3 (IRF3). However, some cell lines showed no induction of NF-κB or IRF3; instead, TLR3-dependent cell death was induced in these cell lines. However, in the report [10] by Khvalevsky and co-workers, higher levels of poly (I:C)-induced apoptosis were observed in a colon adenocarcinoma cell line, HepG2, then in a hepatoma cell line, Huh7, or in a human embryonic kidney cell line, HEK293, which suggested that sensitivity to poly (I:C) might be cell-species specific. One explanation for such a difference in apoptotic sensitivity was suggested by Meylan et al. [18], who previously noted that RIP1 activity was required in TLR3 signaling, which indicated that in some cell species, the ability of RIP1 to induce caspase activation was impaired.

With regard to the involvement of Akt in TLR3 signaling, Sarkar et al. [19] revealed that the PI3K-Akt pathway was crucial for TLR3-mediated double-strand RNA-induced genes such as ISG56. Sarkar and colleagues demonstrated that TLR3 downstream of IRF3 was not fully phosphorylated when recruitment of PI3 kinase to TLR3 was blocked, suggesting an essential role for the PI3K-Akt pathway in the TLR3-mediated innate response. Inhibition of PIK3 by a specific inhibitor, LY294002, was followed by clearly impaired TLR3-mediated signaling. In our experiment, phosphorylation of Akt was accompanied by poly (I:C)-induced apoptosis of SGECs. It remains unclear why the phosphorylation of Akt was correlated with the apoptotic process; however, the downstream signal following the adaptation of TRIF to TLR3 might be involved in the phosphorylation of Akt, coupled with RIP1 activation, which is known to lead to the cleavage of caspase.

Proapoptotic signals in pSS have been reported in previous studies of Fas/Fas ligands, i.e., granzyme/perforin cytotoxic granules generated by CD8+ cytotoxic T lymphocytes [20–24]. As we have reported recently, in cultured SGECs, the Fas/Fas ligand system is well understood [7, 8], and the Fas signal accompanying PI3K inhibition is known to have the potential to induce apoptosis. Furthermore, we recently revealed a rapid induction of apoptosis by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in the SGECs of pSS [8]. As regards TLR3-mediated cell death in patients with pSS, Manoussakis et al. [25] recently reported that detachment-induced apoptosis was observed in poly (I:C)-treated SGECs from patients with SS. However, the innate immunity-related induction of apoptosis and the anti-apoptotic system in pSS has not yet been fully elucidated. Although no direct association with SS was observed, Numata et al. [26] more recently demonstrated TLR3-mediated apoptosis of human bronchial epithelial cells. Their study clearly showed that insulin-dependent PI3K-Akt signaling inhibited TLR3-mediated cell death. Thus, their results might help elucidate the role of the PI3K-Akt pathway as an anti-apoptotic process in TLR3-mediated cell death. Here, another possibility except TLR3 to induce apoptosis can be considered because TLR3

expression was observed after 24-h stimulation with poly (I:C). Since poly (I:C) also strongly induced melanoma differentiation-associated gene-5 or retinoic acid-induced protein I [27], these mechanisms should be concerned in the apoptotic process of SGEC in SS. The present study suggests a new mechanism to account for salivary gland cell death. The detailed relationship between PI3K-Akt signaling and molecules downstream of the ligation with TLR3 remains supported by the evidence, since PI3K-Akt appears to act as an inducer of the poly (I:C)-induced apoptotic cell death of SGECs. However, we should note that poly (I:C)-induced expression of TLR3 and phosphorylated Akt in pSS patients and a normal subject were similar. Since the difference was found in poly (I:C)-induced MAP kinases and apoptosis, the phenomenon might explain difference of sensitivity toward pro-apoptotic signal in both groups. Phosphorylation of MAP kinases induced by poly (I:C) is different due to cell species or time course. For example, phosphorylation of p38 is found in corneal fibroblasts at 60-min stimulation with poly (I:C) [28].

In summary, we focused on TLR3-induced apoptosis and the associated phosphorylation of Akt in pSS. These findings may provide novel insights into the apoptotic and anti-apoptotic systems found in the labial salivary glands in pSS. However, the precise signals downstream of TLR-3 have yet to be determined. Downstream signal analysis and related investigations will be necessary to elucidate TLR3-mediated apoptosis of SGECs in pSS.

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Selective localization of T helper subsets in labial salivary glands from primary Sjögren's syndrome patients

T. Maehara, M. Moriyama, J.-N. Hayashida, A. Tanaka, S. Shinozaki, Y. Kubo, K. Matsumura and S. Nakamura
Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, Fukuoka, Japan

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Correspondence: S. Nakamura, Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: seiji@dent.kyushu-u.ac.jp

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Summary

The aim of this study was to investigate the initiation and progression of autoimmune damage in the lesions of labial salivary glands (LSGs) from primary Sjögren's syndrome (SS) patients by examining the selective localization of T helper (Th) subsets such as Th1, Th2, Th17 regulatory T cells (T_{regs}) and follicular T helper cells (Tfh). The expression of cytokines and transcription factors associated with these Th subsets in the LSGs from 54 SS patients and 16 healthy controls was examined using real-time polymerase chain reaction (PCR) and immunostaining. Additionally, infiltrating lymphocytes without germinal centre (GC^-) and with GC (GC^+) in the LSGs specimens from eight SS patients were extracted selectively by laser capture microdissection (LCM). The mRNA expression of these molecules was compared between the two sample groups of GC^- and GC^+ by real-time PCR. The mRNA expression of cytokines and transcription factors of all T helper (Th) subsets in the LSGs from the SS patients was increased significantly in comparison with controls. In LSGs from the SS patients, Th2 and Tfh was associated closely with strong lymphocytic infiltration; however, Th1, Th17 and T_{regs} was not. In the selectively extracted lesions of LSGs, Th1 and Th17-related molecules were detected strongly in the GC^-, while Th2 and Tfh-related molecules were detected in the GC^+. In contrast, no significant association with strong lymphocytic infiltration was observed in T_{reg}-related molecules. These results indicate that SS has selective localization of Th subsets such as Th1, Th2, Th17 and Tfh in the LSGs, which is associated closely with disease severity and/or status. SS might be initiated by Th1 and Th17 cells, and then progressed by Th2 and Tfh cells via GC formation.

Keywords: cytokine, laser capture microdissection, Sjögren's syndrome, T helper subsets, transcription factor

Introduction

Sjögren's syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration into the salivary and lacrimal glands with concomitant destruction of the glandular tissue and autoantibody production. In approximately 5% of patients the disease may progress to the development of B cell lymphoma, possibly accompanied by hypergammaglobulinaemia and immunodeficiency [1], in close association with B cell accumulation [2,3] and germinal centre (GC) formation [4] in salivary glands. Histologically, SS is characterized by extensive lymphocytic infiltration of the salivary glands [5], and the majority of infiltrating cells are T cells, predominantly $CD4^+$ T cells but also $CD8^+$ T cells, B cells, plasma cells and macrophages [6]. Although it is generally thus accepted that $CD4^+$ T helper (Th) cells play a crucial role in the pathogenesis of SS, the pathological role of $CD4^+$ T cells in SS remains to be elucidated.

Several studies indicate that the Th cell population is comprised of functionally distinct subsets characterized by specific patterns of cytokines and transcription factors [7,8], and that at least six subsets exist – Th0, Th1, Th2, Th17,

regulatory T (T_{reg}) and follicular helper T (Tfh) cells. Th1 cells induced by interleukin (IL)-12 are mainly responsible for cell-mediated immunity, while Th2 cells induced by IL-4 are responsible for humoral immunity. These subsets are then controlled mutually by their own cytokines. Several studies have reported that some autoimmune diseases or allergic diseases are caused by the collapse of Th1/Th2 balance. In contrast, Th0 cells are produced by both Th1 and Th2 cytokines and are considered to be precursors of Th1 and Th2 cells. T_{reg} cells are essential for the maintenance of immunological self-tolerance and immune homeostasis. Furthermore, Th17 cells have been shown to play a crucial role in the induction of autoimmunity and allergic inflammation [9]. Recently, Tfh cells contribute to impaired B cell differentiation and humoral immunity in conditions of immunodeficiency [7]. These Th subsets are generally considered to maintain the balance and homeostasis of the immune system, and possibly to induce various diseases by their impaired regulation. Importantly, the unique interactions and cross-talk between these Th subsets appear to be involved intimately in autoimmunity [8,10–12]. Consequently, an understanding of the presence and distribution of local Th subsets in the salivary glands from SS patients is relevant in oreder to clarify the mechanisms of the onset and progression of SS.

As glandular lymphocytic infiltration is a progressive feature in SS, in some patients ectopic GC can occur in the labial salivary glands (LSGs) [13]. Ectopic GC was defined as B and T cell follicles with follicular dendritic cell networks, high endothelial venules and clusters of proliferating cells. SS patients with ectopic GC in the LSGs coincided with aberrations of serum anti-Ro/SSA, anti-La/SSB and immunoglobulin (Ig)G levels in comparison with without GC [14]. Ectopic GC formation in the LSGs was involved in the pathogenesis of SS [15]. GCs have been recognized as important loci for the maturation of B cells and the generation of B cell lymphomas [16]. Furthermore, we have reported that Th2 cells are involved in the progression of the disease process in SS, especially local B cell activation [17]. Recently, Theander *et al.* [4] reported that the detection of GC-like structures (B cell accumulation) in the LSGs biopsy specimens from primary SS patients is proposed as a highly predictive and easy-to-obtain marker for B cell lymphoma development. Therefore, elucidating the mechanisms leading to ectopic GC formation may be of critical importance in clarifying further the pathogenesis of the disease and the possible development of salivary gland lymphomas. However, we are aware of no published reports that describe ectopic GC formation and the selective localization of Th subsets at any lesions in SS. We thus focused on the infiltrating lymphocytes, especially Th subsets, around ductal epithelial cells and ectopic GC in the LSGs, and adopted a laser capture microdissection (LCM) technique to obtain tissue samples exclusively from specific regions of interest [18,19].

Patients and methods

Patients

Fifty-four patients with primary SS (51 female and three male) referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital, which is a tertiary care centre, between 2007 and 2011 were included into the study. The patients ranged in age from 21 to 88 years [mean ± standard deviation (s.d.) age: 61·2 ± 11·5 years]. Medical records were reviewed retrospectively after diagnosis. All fulfilled the diagnostic criteria for definite SS proposed by the Research Committee on SS of the Ministry of Health and Welfare of the Japanese Government (1999) [20], and the diagnosis was also based on the diagnostic criteria proposed by the American–European Consensus Group criteria for SS [21]. Each patient exhibited objective evidence of salivary gland involvement based on the presence of subjective xerostomia and a decreased salivary flow rate, abnormal findings on parotid sialography and focal lymphocytic infiltrates in the LSGs. There was no documented history of treated with steroids, human immunodeficiency virus (HIV), infection with hepatitis B virus, hepatitis C virus, sarcoidosis and any other immune depressants in any of the patients. None of the patients with SS had evidence of malignant lymphoma at the time of the study. The prevalence of anti-SS-A/Ro, anti-SS-B/La and anti-nuclear antibodies were, respectively, 75·7, 31·2 and 78·2%. The LSG biopsies were performed as described by Greenspan *et al.* [22].

Sixteen patients with mucoceles (14 female and two male) who had no clinical or laboratory evidence of systemic autoimmune disease for use were chosen as a control group. The patients ranged in age from 37 to 66 years (mean age: 52·3 years; 56·8 ± 16·3 years). All control LSGs were histologically normal.

Informed consent, which was approved by the Ethics Committee of Kyushu University, Japan, was obtained from all the patients and healthy controls included in the study.

Histological study of LSGs

A histological study was performed in the LSG specimens from 54 patients in whom enough LSGs were available. Four-μm formalin-fixed and paraffin-embedded sections of LSG specimens were prepared and stained with haematoxylin and eosin (H&E) for conventional histological examination. The degree of lymphocytic infiltration in the specimens was judged by focus scoring [22,23]. One standardized score is the number of focal inflammatory cell aggregates containing 50 or more mononuclear cells in each 4-mm^2 area of salivary gland tissue [24]. Ectopic GC are defined only by H&E stainings as a well-circumscribed chronic inflammatory cell infiltrate consisting of at least 50 mononuclear cells presenting with a densely packed

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dark zone and less densely packed light zone, as described previously by Jonsson *et al.* [14]. Only a few of these structures defined as such correspond, in fact, to real GC.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was prepared from the whole LSGs by the acidified guanidinium–phenol–chloroform method, as described previously [25,26]. Three micrograms of the total RNA preparation was then used for the synthesis of cDNA. Briefly, RNA was incubated for 1 h at 42°C with 20 U of RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), 0·5 μg of oligo-(dT) 1218 (Pharmacia, Uppsala, Sweden), 0·5 mM of each deoxyribonucleotide triphosphate (dNTP) (Pharmacia), 10 mM of dithiothreitol (DTT) and 100 U of RNase H reverse transcriptase (Life Technologies, Gaithersburg, MD, USA).

Tissue sampling by LCM

LCM was performed using a Leica Microsystems Japan (AS-LMD; Leica Microsystems Japan, Tokyo, Japan), as described previously [18,19]. In preparation for LCM, 13-μm-thick frozen sections were cut from these LSGs. The sections were first treated with 0·5 M ethylenediamine tetraacetic acid (EDTA) for 3 min, dehydrated with graded concentrations of ethanol, stained with HistoGene Staining Solution [LCM Frozen Section Staining Kit (Arcturus®; Applied Biosytstems, cat. no. KIT0401NS, Foster City, CA, USA)] for 45 s. After washing in distilled water, they were dehydrated with 100% ethanol, then clarified with xylene and air-dried with a fan for 30 s. All reagents were prepared RNase-free, and the entire process was completed within 20 min to minimize RNA degradation. Briefly, the tissue area of interest was positioned and cut out using a focused pulsed laser beam. Dissected areas were collected in the cap of a microcentrifuge tube via laser pressure catapulting. The cap was filled with 30 μl QIAzol lysis reagent (Qiagen, cat. no. 79306, Valencia, CA, USA).

RNA extraction from microdissected samples and cDNA synthesis

Total RNA was extracted independently from the LCM samples by using the RNeasy Mini Kit (Qiagen). Three micrograms of the isolated total RNA preparation was then used for the synthesis of cDNA, as described previously [25,26].

To determine the minimum amount of cells necessary to obtain enough RNA for a stable real-time quantitative polymerase chain reaction (PCR), infiltrating lymphocytes in the LSG specimens were microdissected and used for RNA isolation, cDNA synthesis and real-time PCR. For quantification with real-time PCR, it is recommended not to use an amplification crossing-point over 40 cycles. Applying this rule, for quantification purposes at least 15 frozen sections for each patient are recommended, depending on cell density. mRNA expression for all experiments was detected by PCR (date not shown).

Quantitative estimation of mRNA by real-time PCR

Quantitative cDNA amplification from the whole LSGs and the microdissected samples was performed according to the manufacturer's instructions and previous reports [25,26]. The cDNA of the cytokines and transcription factors were analysed by real-time PCR using Light Cycler Fast Start DNA Master SYBR Green 1 (Roche Diagnostics, Mannheim, Germany) in a Light Cycler real-time PCR instrument (version 3·5; Roche Diagnostics). Each Th subset expressed transcription factors T box transcription factor (T-bet), GATA3, retinoic acid-related orphan receptor C2 (RORC2) [27], forkhead box protein 3 (FoxP3) and B cell lymphoma 6 (Bcl-6), representing Th1, Th2, Th17, T_{reg} and Tfh cells, respectively [8]. In this study, the cytokines and transcription factors examined were IL-4, IL-5, IL-10, IL-12, IL-17, IL-21, interferon (IFN)-γ, transforming growth factor (TGF)-β, T-bet, GATA3, RORC2, FoxP3 and Bcl-6.

In order to provide a meaningful comparison between different individuals or samples, we calculated the relative amounts of the PCR products of these molecules to the amounts of β-actin PCR products (for the standardization of total cellular mRNA) in each sample, as described previously [17,26].

Immunohistochemical analysis

For immunohistochemical analysis, 4-μm formalin-fixed and paraffin-embedded sections were prepared and stained by a conventional avidin–biotin complex technique, as described previously [26,28]. The polyclonal antibodies used to analyse the cytokines were anti-IL-4 (clone: ab9622; Abcam, Cambridge, UK), anti-IFN-γ (clone: ab9657; Abcam), anti-IL-17 (clone: sc-7927; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-IL-21 (LS-C401; LifeSpan BioScience, LSBio, Seattle City, WA, USA) were used as control rabbit polyclonal antibodies. The mouse monoclonal antibodies used to analyse transcription factors were anti-FoxP3 (clone: mAbcam 22510; Abcam), anti-Bcl-6 (clone: ab9479; Abcam) and anti-CXC chemokine receptor 5 (CXCR5) (clone: ab89259; Abcam). The sections were incubated sequentially with primary antibodies, biotinylated anti-mouse IgG secondary antibodies (Vector Laboratories, Burlingame, CA, USA), avidin–biotin–horseradish peroxidase complex (Vector Laboratories) and 3,3′-diaminobenzidine (Vector Laboratories). Mayer's haematoxylin was used for counterstaining. Photomicrographs were obtained using a light microscope equipped with a digital camera (CoolSNAP; Photometrics, Tucson, AZ, USA).

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Statistical analysis

The statistical significance of the differences between the groups was determined by the Mann–Whitney *U*-test. All statistical analyses in this study were performed using JMP software (version 8; SAS Institute, Tokyo, Japan). A *P*-value of less than 0·05 was considered statistically significant.

Results

The mRNA expression levels of cytokines and transcription factors detected in whole LSGs

mRNA expression levels of IL-4, IL-5, IL-10, IL-17, IL-21, IFN-γ, T-bet, GATA3, RORC2, FoxP3 and Bcl-6 in whole LSGs from the 54 SS patients were significantly higher than those from the 16 controls (Fig. 1a). The 54 SS patients were then divided into two groups: one group of patients with weak lymphocytic infiltration of LSGs ($n = 26$; focus score range: 1–6; mean ± s.d., 3·1 ± 1·9) and a second group of patients with strong lymphocytic infiltration ($n = 28$; focus score range 7–12; mean ± s.d., 8·6 ± 2·2). mRNA expression levels of IL-4, Bcl-6 and GATA3 in whole LSGs with strong lymphocytic infiltration were significantly higher than in those with weak lymphocytic infiltration (Fig. 1b). In contrast, the mRNA expression levels of IL-5, IL-10, IL-12, IL-17, IL-21, IFN-γ, TGF-β, T-bet, RORC2 and FoxP3 in whole LSGs showed no relationship to the degree of lymphocytic infiltration.

Histological study and LCM of the LSGs

The histological findings were examined in the LSG specimens from the 54 SS patients in whom enough LSGs were available. Formalin-fixed LSGs from these patients were screened for the ectopic GC. Among the screened patients,

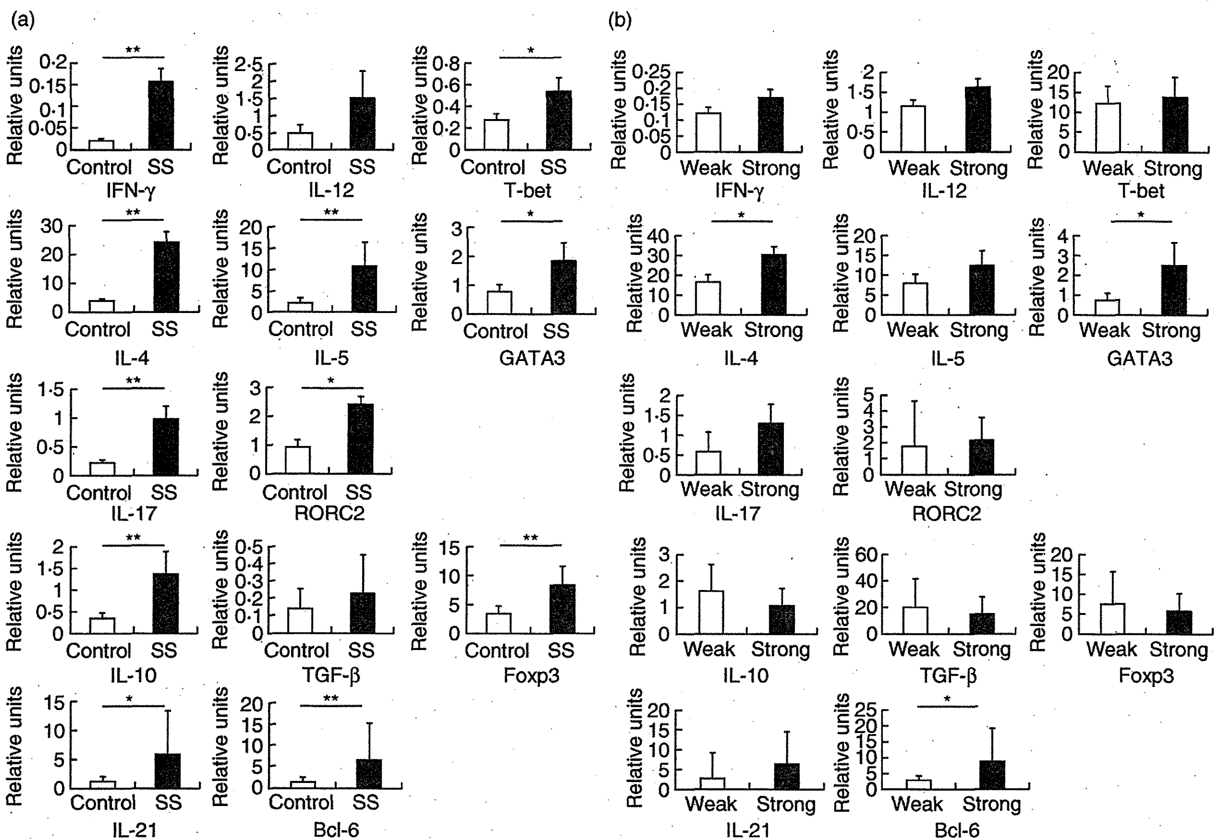

Fig. 1. (a) mRNA expression levels of cytokines and transcription factors were compared in the whole labial salivary glands (LSGs) from Sjögren's syndrome (SS) patients ($n = 54$) and controls ($n = 16$). T helper type 1 (Th1): interferon (IFN)-γ, interleukin (IL)-12 and T-bet; Th2: IL-4, IL-5 and GATA3; Th17: IL-17 and retinoic acid-related orphan receptor C2 (RORC2); regulatory T cells (T_{regs}) type: IL-10, transforming growth factor (TGF)-β and forkhead box protein 3 (FoxP3); Tfh type: IL-21 and B cell lymphoma 6 (Bcl-6) were estimated quantitatively, as described in the Patients and methods section. (b) mRNA expression levels in whole LSGs from SS patients were associated with the degree of lymphocytic infiltration, scored as weak ($n = 26$) or strong ($n = 28$). Bars represent means and standard deviations. Significant differences between groups were determined by Mann–Whitney *U*-tests (*$P < 0.05$; **$P < 0.01$).

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the 12 SS patients (22%) were found to have the structures. For diagnostic purposes, at least five minor salivary glands were recommended [13]. All the LSGs from SS patients showed periductal lymphocytic infiltration with atrophy or severe destruction of the acini. Eight of our 12 SS patients had ectopic GC formation in the frozen LSGs. The remaining four SS patients did not have this structure. We thus selected frozen LSGs with ectopic GC formation from the eight SS patients, the clinical characteristics of which are summarized in Table 1. These frozen specimens from the eight SS patients were available for LCM. In these LSG specimens, the infiltrating lymphocytes in/around ducal epithelial cells without ectopic GC were defined as GC⁻, while with ectopic GC were defined as GC⁺ (Fig. 2a). As shown in Fig. 2b, GC⁻ and GC⁺ from the same LSGs specimens were isolated by LCM. Total RNA was isolated independently from the LCM samples. Isolated total RNA was reverse-transcribed to generate cDNA. As mRNA expression isolated by LCM is a very small quantity (20–50 ng/μl), it is detected by PCR (Fig. 2c).

Comparison of the mRNA expression levels of cytokines and transcription factors between GC⁻ and GC⁺

As shown in Fig. 3a,c, the mRNA expression of IL-12, IL-17, IFN-γ, T-bet and RORC2 in the GC⁻ was significantly higher than that in the GC⁺. As shown in Fig. 3b,e, the mRNA expression of IL-4, IL-21, GATA3 and Bcl-6 in the GC⁺ was significantly higher than that in the GC⁻. As shown in Fig. 3d, the mRNA expression of IL-10, TGF-β and FoxP3 showed no statistically significant difference.

Immunohistochemical analysis of cytokines and transcription factors in the LSGs

As shown in Fig. 4a, the specimens were examined immunohistochemically to evaluate the distributions of these proteins in the LSGs from SS patients and healthy controls. Expression of IFN-γ and IL-17 was detected slightly in ductal epithelial cells in LSGs from healthy controls (Fig. 4a,e), but was detected more strongly in/around the ductal epithelial cells rather than the ectopic GC from SS patients (Fig. 4b–d,f–h). Expression of IL-4 was detected in acinar cells in LSGs from SS patients and healthy controls (Fig. 4i,j,k), but was detected more strongly in/around the ectopic GC rather than in/around the ductal epithelial cells from SS patients (Fig. 4j–l). Expression of FoxP3 was not detected in the LSGs from healthy controls (Fig. 4m), but was detected in the diffuse-infiltrating lymphocytes of LSGs from SS patients (Fig. 4n–p). However, expression of FoxP3 in LSGs from SS patients showed no difference between GC⁻ and GC⁺. Expression of Bcl-6 was not detected in LSGs from healthy controls (Fig. 4q), but was detected in/around the ectopic GC (Fig. 4r–t). As shown in Fig. 4b, expression of Bcl-6, IL-21 and the chemokine receptor CXCR5 in Tfh was

Table 1. Clinical characteristics of eight patients (focus score 7–12) with primary Sjögren's syndrome (SS).

No.	Age (years)	Sex	Lymphocytic infiltration†	Autoantibody					Immunoglobulin		
				RF (20–≥ U/ml)	ANA (titre)	DNA (10–> U/ml)	SS-A/Ro (10–>)	SS-B/La (15–>)	IgG (872–1815 mg/dl)	IgA (95–405 mg/dl)	IgM (59–269 mg/dl)
1	26	F	10	25	> 1280	ND	> 256	> 15	> 1815	Negative	Negative
2	44	F	9	< 20	Negative	Negative	104	29	1517	193	174
3	65	F	9	n.d.	80	n.d.	64	< 15	n.d.	n.d.	n.d.
4	52	F	11	40	> 1280	< 5	> 256	32	2206	270	107
5	48	F	10	35	320	< 5	16	< 15	n.d.	n.d.	n.d.
6	65	F	9	n.d.	160	2	< 10	< 15	1338	n.d.	62
7	53	F	11	n.d.	n.d.	n.d.	< 10	< 15	n.d.	n.d.	n.d.
8	59	F	12	n.d.	n.d.	n.d.	> 256	< 15	n.d.	n.d.	n.d.

†The degree of lymphocytic infiltration in the labial salivary glands (LSGs) was graded from 1 to 12 by focus scoring. Ig: immunoglobulin; F: female; n.d.: not done; RF: rheumatoid factor; ANA: anti-nuclear antibodies.

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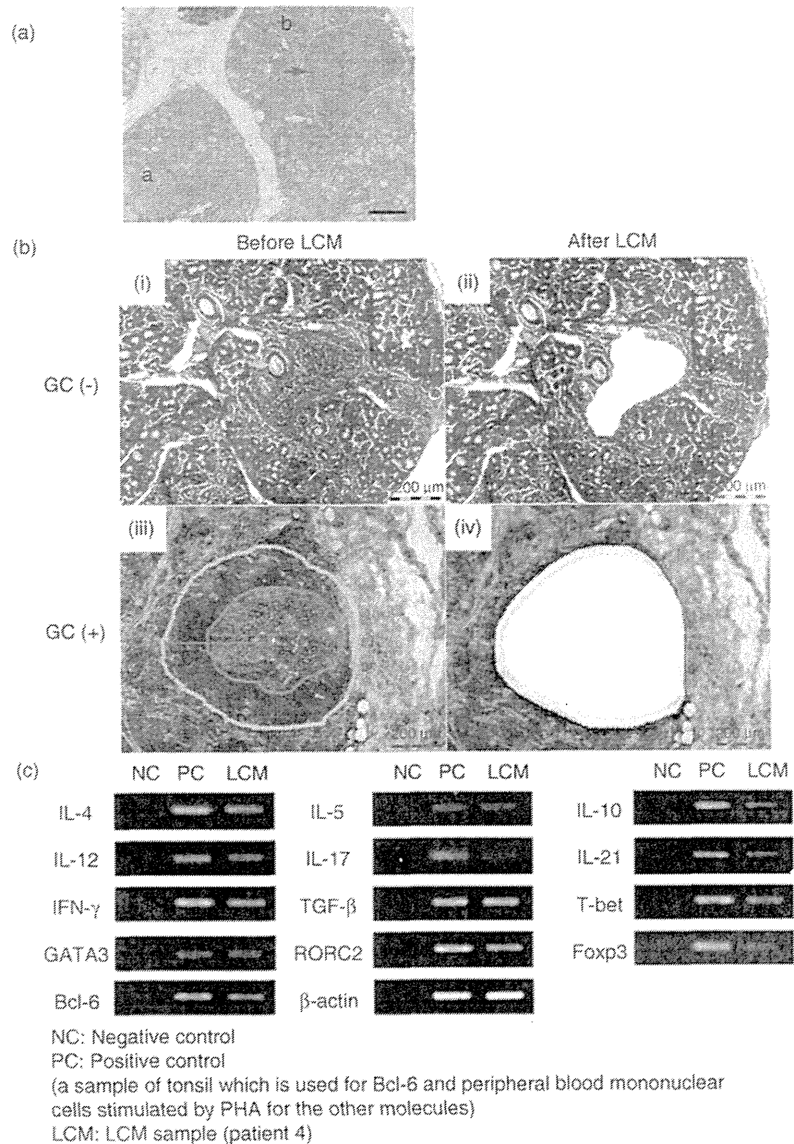

Fig. 2. Representative results from patient 4. (a) Haematoxylin and eosin-stained labial salivary glands (LSGs). (i) Lymphocyte infiltration (enclosed by red broken line) around the ducts (yellow arrow). (ii) Secondary lymphoid follicles (enclosed by yellow broken line) show lymphocytic infiltration around the ectopic germinal centre (GC) (blue arrow) and the ectopic GC (enclosed by green broken line). Scale bars, 100 μm (original magnification ×100). (b) LSG specimens before/after laser capture microdissection (LCM). A defined area of infiltrating lymphocytes was marked using AS LMD microdissection software (Leica Microsystems) before LCM (i, iii). The preselected area was microdissected using a guided laser beam after LCM (ii, iv). The yellow arrow shows ductal epithelial cells (i–iv). (c) Secondary lymphoid follicles (enclosed by yellow line) showing lymphocytic infiltration around the ectopic GC, and the ectopic GC (enclosed by green broken line). Infiltrating lymphocytes in/around the GC were collected from a distance of approximately 1·5 GC radii (r) by LCM. Care was taken to avoid capturing epithelial cells (ii, iv). Scale bars, 200 μm (original magnification ×200). (c) Polymerase chain reaction (PCR) products from LCM specimens.

detected in/around the ectopic GC in the LSGs from the same SS patients. Expression of CXCR5 was detected especially around the ectopic GC (Fig. 4u–w).

Discussion

The findings of this study are: (1) the expression of all Th subset-related molecules in whole LSGs from SS patients was higher than those in the controls. (2) The expression of Th2 and Tfh-related molecules was associated closely with strong lymphocytic accumulation in whole LSGs from SS patients. (3) In the selectively extracted lesions of LSGs, expression of Th1- and Th17-related molecules in infiltrating lymphocytes without ectopic GC was higher than those with ectopic GC. In contrast, expression of Th2 and Tfh-related molecules in infiltrating lymphocytes with ectopic GC was higher than in those without ectopic GC.

Analyses over time of the mRNA expression of cytokines and transcription factors in whole LSGs performed neither a prospective cohort study nor multiple biopsies. From a practical viewpoint, it is difficult to evaluate the initiation and progression of SS. The acquisition of lymphoid features by inflammatory foci in the LSGs of SS is associated critically with enlargement of the inflammatory foci and with secondary lymphoid follicles [29]. Therefore, in order to evaluate accurately the propagation of the disease process, in the same SS patients a novel strategy was thus used to compare the expression of cytokines and transcription factors in the infiltrating lymphocytes with/without ectopic GC in the LSGs specimens by using LCM.

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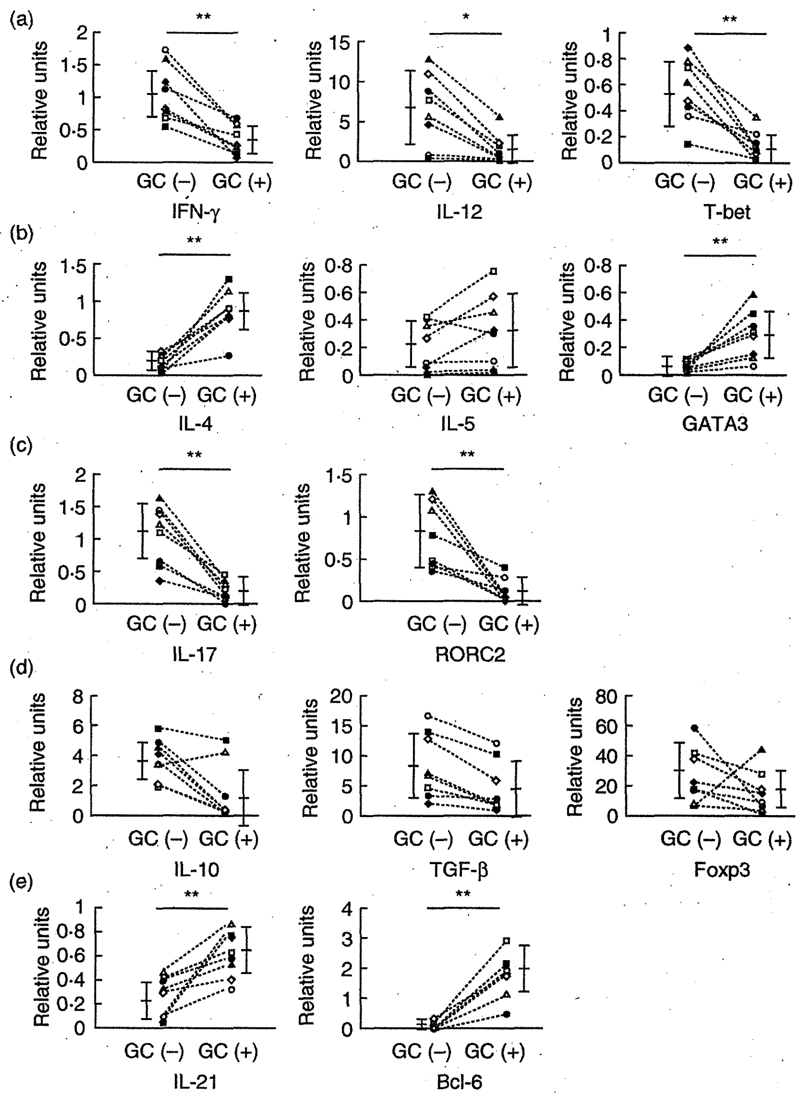

Fig. 3. Comparison of mRNA expression patterns of cytokines and transcription factors in germinal centre (GC)[–] and GC[+] from Sjögren's syndrome (SS) patients ($n = 8$), using laser capture microdissection (LCM). Real-time polymerase chain reaction (PCR) products of interleukin (IL)-4, IL-5, IL-10, IL-12, IL-17, IL-21, interferon (IFN)-γ, transforming growth factor (TGF)-β, T-bet, GATA3, retinoic acid-related orphan receptor C2 (RORC2), forkhead box protein 3 (FoxP3) and Bcl-6 were estimated quantitatively, as described in the Patients and methods section. Bars show means and standard deviations. Significant differences between groups were determined by Mann–Whitney *U*-tests (*$P < 0.05$; **$P < 0.01$).

In SS-susceptible mice, the elimination of Th1 cells ameliorated all pathological and clinical signs of the disease [30]. Our previous study suggested a model of the pathogenesis of SS [17]. The mutual stimulation of Th1 cells and the target organ via the production of various cytokines plays a key role in the induction and/or maintenance of the disease and results in the eventual destruction of the target organ. Recently, CD4^{+} Th17 cells have been shown to be tissue-seeking and involved intimately in the initiation of SS [31,32]. The results of the present study concerning lymphocyte subsets and cytokine production in the LSGs are consistent with this model. Youinou *et al.* [33] reported that Th17 cells orchestrate autoreactive GCs. Our results were consistent with this report. However, in the selectively extracted lesions of LSGs, expression of Th17-related molecules in infiltrating lymphocytes without the ectopic GC was higher than in those with the ectopic GC. Interestingly, Th17/Th1 cells reportedly co-express IFN-γ with IL-17 [34], and such a subset has been identified in the gut in Crohn's disease [35]. Both Th1 and Th17 cells together were involved in the pathogenesis of SS [36], and there was an early induction of a CD4^{+} Th1/Th17 pathway leading to systemic release of IL-17 in mice [31]. Our observations suggest that both Th1 and Th17 cells around the ductal epithelial cells might be of critical importance in the initiation of SS.

Mitsiasis *et al.* [37] have reported that the balance between Th1 and Th2 shifted in favour of the former in LSG with a high infiltration score. Our results are consistent with these results. In our present data, mRNA expression of Th1 cytokines in the LSG with both weak and strong lymphocytic infiltration from SS patients was also significantly higher than that from controls. Therefore, these results suggest that Th1 cytokines play a key role in the induction and maintenance of the disease. Conversely, we reported that the levels

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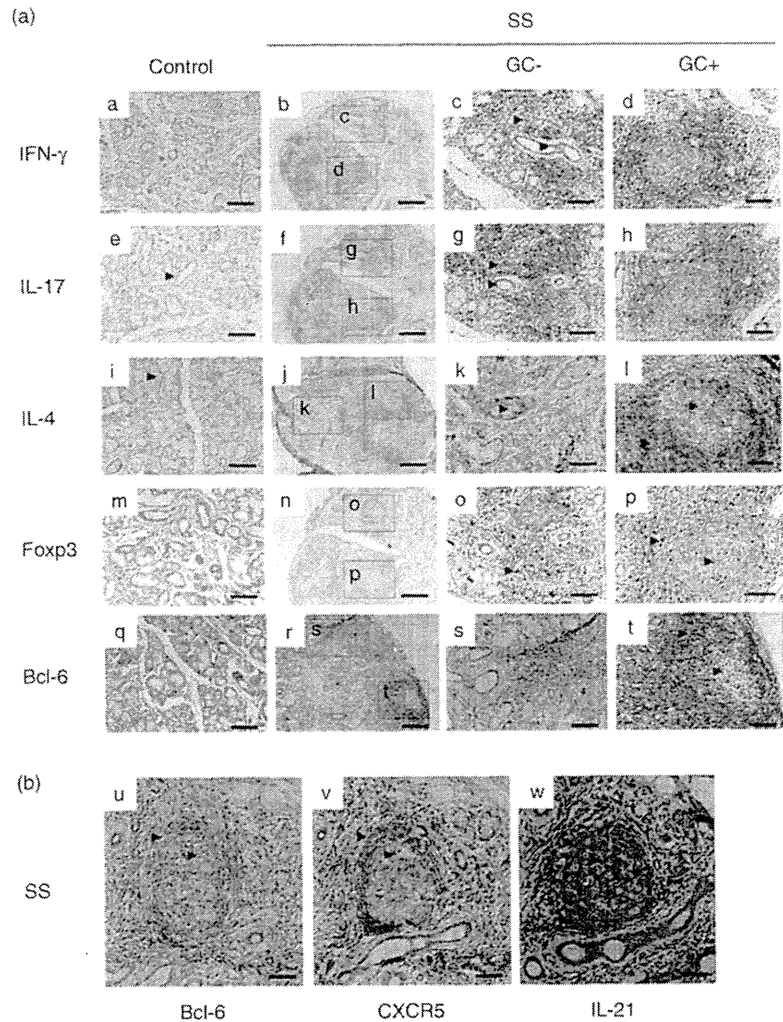

Fig. 4. Distribution of T helper (Th) subsets in the lesions of labial salivary glands (LSGs). Immunostaining with anti-interferon (IFN)-γ (a–d), anti-interleukin (IL)-4 (e–h), anti-IL-17 (i–l), anti-forkhead box protein 3 (FoxP3) (m–p), anti-Bcl-6 (q–t, u) and anti-CXC chemokine receptor 5 (CXCR5) (v) monoclonal antibodies in LSGs from representative patients with Sjögren's syndrome (SS) (b–d, f–h, j–l, n–p, r–t, u, v) and healthy subjects (a, e, i, m, q) (brown). Counterstaining with Mayer's haematoxylin (blue). Arrows indicate key features of infiltrating cells. Representative sections from SS patient 6 are shown. Scale bars, 100 μm (original magnifications ×100, ×200, ×300).

of mRNA for both Th1 and Th2 cytokines and chemokines in LSGs with strong lymphocytic infiltration from patients with SS were significantly higher than in controls [26]. As described above, Theander *et al.* [4] reported that the ectopic GC-like structures, including ectopic GC in the LSGs, was involved in the development of malignant lymphoma in primary SS. We thus speculate that additional Th2 cells play a role in the lympho-aggressiveness of the disease. This paper focused on infiltrating lymphocytes, particularly Th subsets, around the ductal epithelial cells and ectopic GCs in LSGs, using a LCM technique to obtain tissue samples exclusively from specific regions of interest. We have positive results indicating that expression of Th2 cytokines in infiltrating lymphocytes with the ectopic GC was higher than that around the ductal epithelial cells. From the results obtained in this study, we speculated that Th2 cells might be involved in the progression of the disease, especially in the growth and activation of ectopic GC formation. Furthermore, several studies on autoimmune diseases in mice and humans have indicated a pathogenic role for Th1 cells and a possible protective role for Th2 cells [38,39]. Our results, showing that expression of Th1-related molecules in infiltrating lymphocytes with the ectopic GC was lower than without GC, and that of Th2-related molecules with GC was higher than without GC, are consistent with this report. Previous studies have reported that Th2 effector cells was important role for GC formation [40], and that Th2 cytokines induced infiltrating B lymphocytes to produce autoreactive antibodies [41]. Furthermore, the salivary gland environment in SS, in association with tissue trophic viruses such as the Epstein–Barr virus [42], cytomegalovirus [43] and retrovirus [44], might increase the risk of pseudolymphoma and hypergammaglobulinaemia promotion, and might hasten the progression to B cell malignant lymphoma. Considering the possible role of Th2 cells in the induction of B cell abnormalities, these cells might have a harmful (rather than a protective) effect on SS. Conversely, several studies have reported that expression of the chemokine receptor CXCR5 allows Tfh to home

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to the B cell follicle [45], IL-21 was increased in serum from SS patients [46] and high levels of IL-21 receptor were present at the surface of most B cells [7]. Furthermore, mice that lack the receptors for both IL-4 and IL-21 have greatly reduced IgG responses, indicating that IL-21 co-operates with IL-4 to regulate humoral immune responses [47]. In the LSG lesions, Tfh cells in infiltrating lymphocytes in/around the ectopic GC was significantly higher than that around the ductal epithelial cells (Figs 3 and 4). Our results strongly support that Th2 and Tfh cells are involved in the progression of the disease process as a lympho-aggressive disorder, particularly growth and activation of the ectopic GC formation.

Kolkowski *et al.* revealed that salivary glands in SS consistently express IL-10 and TGF-β [17,48]. Our results obtained in the present study are in accord with this report. Other studies have reported that the remarkable reduction of T_{reg} in LSGs and peripheral blood might be involved in the pathogenesis of salivary gland destruction in SS [49]. Contrary to this study, Gottenberg *et al.* reported that T_{reg} cell numbers were increased in the peripheral blood of patients with SS [50]. Therefore, it is unclear whether or not T_{regs} are involved in the pathogenesis of SS. Recently, the immunoregulatory role of $CD4^{+}CD25^{+}$ T cells might be different at each stage of the disease process in rheumatoid arthritis [51]. However, our results indicated that FoxP3 mRNA expression showed no relationship to the degree of lymphocytic infiltration in whole LSGs from SS patients, between weak and strong lymphocytic infiltration (Fig. 1b). The lower levels of T_{reg} cells in/around the ectopic GC might not result in ectopic GC formation (Figs 3 and 4). Furthermore, recent studies, in which SS patients were divided into three groups (mild, intermediate and severe lymphocytic infiltration in the LSGs), reported that $FoxP3^{+}$ T_{reg} cells at the LSGs of severe SS stage was reduced in comparison with that of intermediate stage SS [3,52]. Our previous study, which concerned the frequency of reduced T_{reg} in the severe stage of LSGs from SS patients, is consistent with this report [26]. Furthermore, increased levels of T_{reg} cells in the whole LSGs from SS patients might suggest that negative feedback is more active in the LSGs from SS patients than in healthy subjects (Fig. 1a). Therefore, these results suggest that T_{regs} might be not involved in the initiation, growth and activation of ectopic GC formation of SS. However, the immunoregulatory role of T_{reg} might be different at each stage of disease status in SS [52]. Conversely, we have also investigated whether Mikulicz's disease, which apparently differs from SS, was a unique IgG4-related disease characterized by Th2 and regulatory immune reactions [26].

Furthermore, we also examined the mRNA expression of Th1-, Th2-, Th17- and T_{reg}-related molecules in peripheral blood mononuclear cells (PBMCs) from SS patients and controls. The mRNA expression of IFN-inducible protein 10 (IP-10) and CXCR3 in PBMCs from SS patients was slightly higher than that from controls, but that of the other molecules was not. In addition, the mRNA expression of all these molecules in the PBMCs showed no significant relationship to the degree of lymphocytic infiltration in the LSGs from the SS patients. These results indicate that few T cells involved in the pathogenesis of SS circulate in the periphery (manuscript in preparation).

In conclusion, we provide new evidence concerning the selective localization of Th subsets in LSGs from SS patients. In addition, it is still necessary to elucidate the mechanisms underlying Th2 and Tfh cell induction to provide functional evidence on the direct role of Th2 and Tfh cell progression, which might lead eventually to the creation of therapeutic strategies for inhibiting the disease progression. A more thorough understanding of the complex mechanisms of the disease, especially those involved in these Th subsets, might lead to pharmacological strategies to disrupt the cytokine network as a further means of inhibiting the initiation and/or progression of SS as a lympho-aggressive disorder.

Although our present study focused on Th subsets, B cells also play a key role in the formation of ectopic GC, and we are thus trying to evaluate the progression of SS more thoroughly (manuscript in preparation).

Acknowledgements

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Disclosure

The authors have declared no conflicts of interest concerning this paper.

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