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Presence of intraepithelial B-lymphocytes is associated with the formation of lymphoepithelial lesions in salivary glands of primary Sjögren's syndrome patients

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Key words: Sjögren's syndrome, lymphoepithelial lesions, histopathology, parotid gland, labial gland

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ABSTRACT

Objective. Lymphoepithelial lesions (LELs) in salivary glands are associated with primary Sjögren's syndrome (pSS). LELs are composed of hyperplastic epithelium infiltrated with lymphocytes. The objective of this study was obtaining insight in the relative roles of intraepithelial B- and T-lymphocytes in the formation of LELs in salivary glands of pSS patients.

Methods. Parotid and labial salivary gland biopsies of pSS patients (n=15), non-SS sicca patients (n=5) and non-sicca controls (n=5) were analysed. Serial sections were stained with H&E and for cytokeratin, CD20 and CD3. Striated ducts with lymphocytes, but without hyperplasia, and striated ducts with LELs were identified in H&E and cytokeratin stained sections. LELs were classified in successive stages of severity based on the amount of hyperplasia (stage 1-3). Numbers of B- and T-lymphocytes within striated ducts and LELs were counted in CD20 and CD3 stained sections.

Results. Lymphocyte-containing striated ducts of both salivary glands of all pSS and control patients harboured T-lymphocytes, scattered throughout the ductal epithelium. In contrast, B-lymphocytes were exclusively found in a small fraction (21%) of striated ducts without hyperplasia and in nearly all striated ducts with LELs of pSS patients, but not in controls. In striated ducts with LELs B-lymphocytes were mostly located in the areas of proliferating epithelium. Numbers of B-lymphocytes and B/T-ratios increased significantly with higher severity of LELs. This was even more pronounced in the parotid than in the labial gland.

Conclusion. We conclude there is an association between presence of in-

traepithelial B-lymphocytes and the formation of LELs in salivary glands of pSS patients.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterised by sicca complaints and lymphocytic infiltration of the lacrimal and salivary glands (1). These infiltrates are mostly situated around the striated ducts, forming periductal foci. The major role of the salivary gland in the pathogenesis of the disease is illustrated by the prominent role of histopathology of the salivary gland in the 2016 ACR-EULAR classification criteria (2). These criteria require either a positive salivary gland biopsy or presence of anti-Ro/SSA antibodies to classify a patient as pSS. A biopsy is suggestive for pSS when the focus score (FS) ≥ 1 . FS is defined as the number of foci (clusters of ≥ 50 lymphocytes) in 4 mm² glandular tissue (3). However, the FS has some important limitations and additional histopathological features may be helpful for diagnosis and classification of pSS (4). One of the features associated with pSS is the formation of lymphoepithelial lesions (LELs) within the foci (5). Ihrler *et al.* postulated that LELs develop in a stepwise manner: lymphocytic infiltration within the ductal epithelium with subsequent hyperplasia of ductal basal cells, causing differentiation into a multi-layered stratified epithelium, or even complete obstruction of the striated ducts (5). Interestingly, a serious complication of the disease is the development of non-Hodgkin lymphoma in 5-10% of the patients, mostly of the mucosa associated lymphoid tissue (MALT) type (6). These lymphomas most commonly develop in the parotid gland and are con-

sistently associated with the presence of LELs in the glandular tissue. Both B- and T-lymphocytes may be present in LELs (4, 7). However, the exact roles of B- and T- lymphocytes in LEL formation are unknown.

The aim of the work described here was to quantify the number of B- and T-lymphocytes within striated ducts without hyperplasia and striated ducts with LELs in both labial and parotid gland biopsies of pSS patients and controls, in order to get insight in the role of lymphocyte subtypes in LEL formation.

Materials and methods

Patients

Both parotid and labial salivary gland biopsies were collected of 15 pSS patients and 5 non-SS sicca patients, without MALT lymphoma, non-specific chronic sialadenitis (NSCS), sclerosing chronic sialadenitis (SCS) or other autoimmune diseases. Patient characteristics are provided in Table I. PSS patients fulfilled the 2016 ACR-EULAR criteria. Non-SS sicca patients were defined as patients having sicca complaints, without serum anti-Ro/SSA antibodies and without foci (FS=0) in neither parotid nor labial salivary glands, and who did not use medication that is known to induce sicca symptoms. Furthermore, biopsy material of non-sicca control patients was obtained from diagnostic redundant material. For parotid gland tissue of non-sicca controls (n=5), resection margins from parotidectomies of patients with a Warthin tumour (n=2) or pleomorphic adenoma (n=3) were used. For non-sicca labial gland tissue (n=5), samples from benign retention cyst (mucocoele) resections were taken. The study of pSS patients and non-SS sicca patients was approved by the Medical Research Ethics Committee of the University Medical Centre Groningen (METc2013.066). All pSS and non-SS sicca patients provided informed consent. For the non-sicca controls, informed consent was not required by Dutch Law for Medical Research and by institutional guidelines. No objection against use of redundant tissue was recorded from these non-sicca controls in the institutional record of objection. Patient material was handled according

Table I. Patient characteristics.

	pSS patients (n=15)	Non-SS sicca patients (n=5)	Non-sicca controls, parotid gland (n=5)	Non-sicca controls, labial gland (n=5)
Age*	49.1 (±14.2)	46.7 (±13.5)	59.2 (±12.2)	24.8 (±17.0)
Female	14 (93)	4 (80)	2 (40)	2 (40)
Anti-SSA-positivity	14 (93)	0 (0)	ND	ND
FS parotid gland	1.3 (0.6 – 1.9)	0 (0-0)	ND	ND
FS labial gland	1.6 (0.8 – 2.3)	0 (0-0)	ND	ND
UWS ≤0.1ml/min	10 (67)	4 (80)	ND	ND
OSS ≥5	8 (62)	0 (0)	ND	ND
Schirmer's test ≤5 mm	13 (87)	2 (40)	ND	ND

Data are given as mean (± SD), number (%) or median (IQR). FS: Focus Score; UWS: unstimulated whole saliva; OSS: ocular staining score; ND: not determined. *Age at the time of diagnosis.

to the 'Code of conduct for health research' of the Dutch Federation of Biomedical Scientific Societies (8).

Histochemical and immunohistochemical staining

Formalin fixed (4%), paraffin embedded tissue samples were serially sectioned at 3 µm thickness and deparaffinised. Tissue samples were automatically stained with haematoxylin and eosin (H&E) and manually stained for CD20 (clone L-26, Ventana Roche), CD3 (clone 2GV6, Ventana Roche) and high molecular weight cytokeratin (hmwCK, clone 34βE12, Ventana Roche). For the immunohistochemical staining antigen retrieval was performed (15 minutes, 98°C in EDTA buffer pH 8.0) and endogenous peroxidase was blocked. Primary antibodies were used at a pre-fixed dilutions in 1% BSA-PBS (Ventana Roche) for 75 minutes. After incubation with a poly-HRP-labelled secondary antibody (Thermo Scientific), the primary antibodies were visualised by using DAB (3,3'-diaminobenzidine). The typical spatial distribution of CD3, CD20 and hmwCK staining within tonsillar tissue was used as both a positive and negative control, as the tonsillar epithelium expresses hmwCK and does not express CD3 and CD20 and the tonsillar lymphoid tissue expresses CD3 and CD20, but does not express hmwCK.

Histological analysis

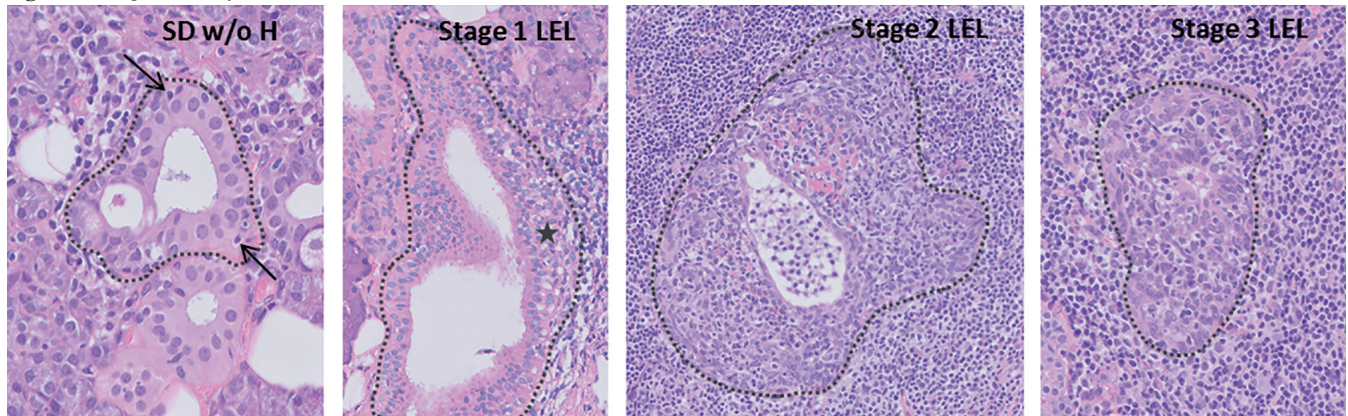
The FS and severity of LELs was scored on H&E stained sections. HmwCK staining was used to identify the ductal epithelium, and to detect "occluded

LELs" (Fig. 1), which are otherwise very difficult to detect unequivocally. Only striated ducts with lymphocytes within the epithelium were analysed. Ducts were divided into two groups: striated ducts with lymphocytes, but without epithelial hyperplasia and striated ducts with lymphocytes with epithelial hyperplasia (*i.e.* LELs). LELs were subdivided into three stages, according to the amount of hyperplasia (Fig. 1). In all biopsies, the 10 most severe LELs were selected for further analysis. In case less than 10 striated ducts with LELs were present, striated ducts without hyperplasia, but with intraepithelial lymphocytes, identified by H&E staining, were selected to complete the total of 10 ducts. If the total number of striated ducts with lymphocytes in the salivary gland tissue was still less than 10, the maximum number of striated ducts with intraepithelial lymphocytes and LELs was analysed. Numbers of B- and T-lymphocytes within each of the striated ducts and LELs were manually scored by using Image J cell counter in serial CD20 and CD3 stained sections.

Statistical analysis

Statistical analyses were performed in IBM SPSS Statistics 23. Generalised estimating equations (GEE) were used to analyse the numbers of B-, T-lymphocytes and B/T-ratios within LELs of pSS patients over the different stages of severity, within striated ducts without hyperplasia of pSS *versus* control patients, and within LELs of parotid *versus* labial glands of pSS patients. Spearman's correlation coefficient was

Fig. 1. Scoring of severity of LELs.



Striated ducts with lymphocytes were divided into two groups: striated ducts without epithelial hyperplasia (SD w/o H) and striated ducts with epithelial hyperplasia (stage 1-3 LELs). Stage 1 LEL: Lymphocytic ductal infiltration and hyperplasia affecting less than 50 percent of the epithelium. Stage 2 LEL: Lymphocytic ductal infiltration and hyperplasia affecting between 50 and 100 percent of the epithelium. Stage 3 LEL: Lymphocytic ductal infiltration and fully circumferentially hyperplastic epithelium without lumen. Arrows point at lymphocytes and the star marks the area of proliferation. Dotted lines mark the ductal border, identified on hmwCK staining.

used to analyse correlations between parotid and labial glands of individual pSS patients and to analyse associations between severity of LELs, FS and clinical features of pSS patients.

Results

Histological analysis

Of the total number of 50 biopsies (25 parotid and 25 labial glands), six biopsies (5 parotid and 1 labial gland) were excluded from the analysis due to insufficient biopsy material (<4 mm²), or because the slides were not stored in a consecutive order. For pSS patients, 11 parotid and 14 labial gland biopsies were analysed, of which 97 and 137 striated ducts were selected for the parotid and labial glands, respectively (median value 10 for both types of glands). For the control group (non-SS sicca patients and non-sicca controls), 9 parotid and 10 labial gland biopsies were analysed, of which 41 and 65 striated ducts were selected (median value 4 and 7, respectively).

Severity of LELs

In pSS patients, striated ducts with LELs were found in 81% of parotid and 86% of labial glands, and they were consistently located in association with periductal infiltrates. This is in contrast to striated ducts with intraepithelial lymphocytes, but without hyperplasia, which were not always associated with periductal infiltrates. Striated ducts with LELs were com-

Table II. Numbers and frequencies of analysed striated ducts without hyperplasia and LELs.

	Parotid gland		Labial gland	
	pSS patients n=99 ducts	Controls n=41 ducts	pSS patients n=137 ducts	Controls n=65 ducts
SD w/o H	63	41	92	65
LELs	36	0	45	0
- Stage 1 LELs	13 (36)	-	33 (73)	-
- Stage 2 LELs	21 (58)	-	9 (20)	-
- Stage 3 LELs	2 (6)	-	3 (7)	-

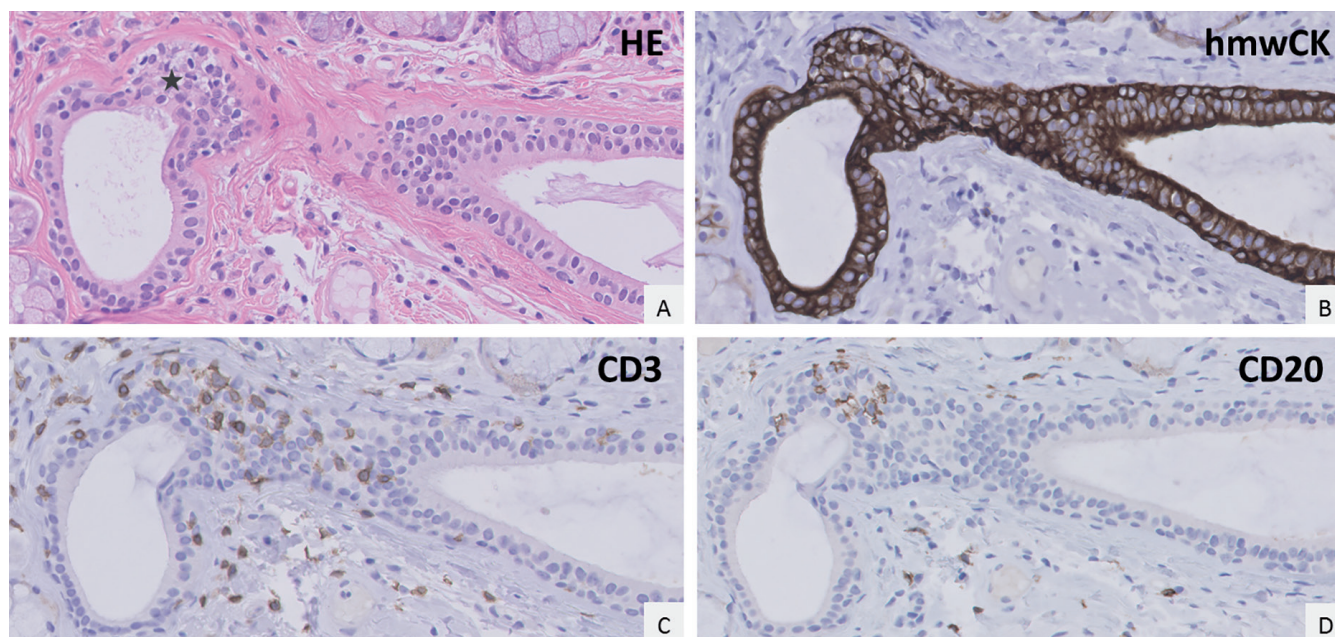
Data are given as number or number (%). SD w/o H: striated duct without hyperplasia; LELs: lymphoepithelial lesions. Controls: non-SS sicca patients and non-sicca controls.

pletely absent in non-SS sicca patients and non-sicca controls; in these patients only striated ducts without epithelial hyperplasia were present. Numbers of analysed striated ducts without hyperplasia and striated ducts with LELs, including frequencies of stages of LELs, are shown in table 2. The number of stage 3 LELs is relatively small in both types of glands, due to the small study population. Stage 2 LELs dominated in parotid glands of pSS patients, whereas stage 1 LELs were most frequent in labial glands. Mean severity of LELs (stage 1-3) of pSS patients was, however, not significantly different between parotid and labials glands (1.38±0.78 and 1.08±0.56, respectively). In the parotid gland of pSS patients, a higher FS was associated with presence of more severe LELs (rs 0.540, p=0.038).

B- and T-lymphocytes within striated ducts and LELs

T-lymphocytes were detected in all

striated ducts without hyperplasia and striated ducts with LELs (stage 1-3) of pSS patients as well as in all striated ducts without hyperplasia of the non-SS sicca patients and non-sicca controls. In contrast, B-lymphocytes were exclusively found in striated ducts without hyperplasia and striated ducts with LELs of pSS patients, and were completely absent in the striated ducts of non-SS sicca and non-sicca controls. B-lymphocytes were nearly always found in striated ducts with LELs of pSS patients, namely in 100% of parotid and 87% of labial gland stage 1-3 LELs. The remaining 13% of LELs without B-lymphocytes in labial glands, were all scored as stage 1 LELs. Interestingly, B-lymphocytes in striated ducts with LELs were mostly concentrated in the areas where the epithelium was proliferating, whereas T-lymphocytes were scattered through the whole ductal epithelium (Fig. 2). Although B-lymphocytes were consist-

Fig. 2. B-lymphocytes are located in the hyperplastic area of the striated duct.

Serial sections were stained with H&E, and for hmwCK, CD3 and CD20. (A) Stage 1 LEL with infiltrating lymphocytes and ductal hyperplasia (star) after H&E staining. (B) Positive hmwCK staining marks the ductal epithelium. (C) CD3⁺ T-lymphocytes are scattered throughout the whole ductal epithelium, whereas (D) CD20⁺ B-lymphocytes are located in the hyperplastic area.

ently found in striated ducts with LELs, they were also present in 21% of striated ducts without hyperplasia of pSS patients; these striated ducts were also associated with a periductal focus.

In the parotid and labial gland of pSS patients, the absolute numbers of B-lymphocytes in the striated ducts with LELs increased significantly with higher severity of LELs (overall increase $p < 0.001$). For T-lymphocytes such an association was only seen in the parotid gland ($p = 0.042$). Absolute numbers of B-lymphocytes showed a higher increase compared to T-lymphocytes, resulting in a significant increase of the B/T-ratio with higher severity of LELs in both glands (overall increase $p < 0.001$) (Fig. 3). In both parotid and labial salivary glands of pSS patients, there was a predominance of T-lymphocytes in striated ducts without hyperplasia as well as in stage 1 LELs (B/T-ratio < 1). In the more severe stages of LELs (stage 2-3), B-lymphocytes outnumbered the T-lymphocytes (B/T-ratio > 1) (Fig. 3). The fact that B-lymphocytes were present in striated ducts without hyperplasia of pSS patients, but not in control patients, was also reflected by a significantly higher number of B-lymphocytes and higher

B/T-ratio in striated ducts without hyperplasia of pSS patients than in non-SS sicca and non-sicca controls in both salivary glands ($p < 0.001$) (Fig. 3).

Numbers of B-lymphocytes, T-lymphocytes and B/T-ratios within LELs (stage 1-3) were significantly higher in the parotid gland than in the labial gland ($p = 0.001$, $p = 0.021$ and $p = 0.007$, respectively). To compare parotid and labial glands of individual pSS patients, the highest number of B-lymphocytes, T-lymphocytes and the highest B/T ratio within LELs of each gland was taken. A correlation was found between the highest number of T-lymphocytes (rs 0.659, $p = 0.038$). No concordance was found between the highest number of B-lymphocytes, highest B/T-ratio and most severe stage of LEL within parotid and labial gland biopsies of individual pSS patients.

Clinical correlations

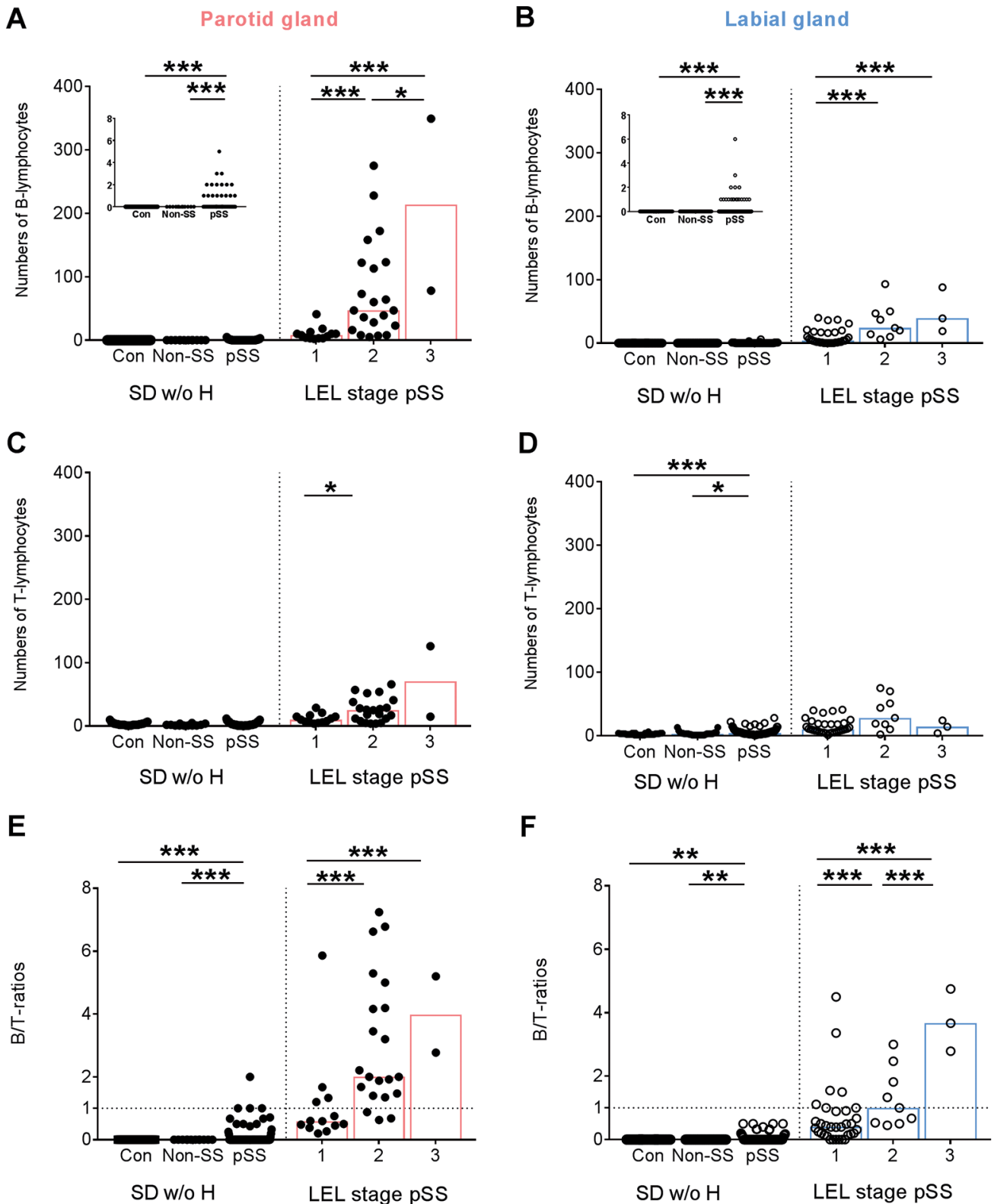
In order to further explore the role of LELs in disease development, severity of LELs was correlated to clinical findings of pSS patients. Positive correlations were found between the highest B/T-ratio in the parotid gland and IgG-levels and rheumatoid factor (RF) levels in plasma of pSS patients (rs 0.790,

$p = 0.004$ and rs 0.696, $p = 0.017$, respectively). A positive correlation was also found between the most severe stage of LEL within labial glands and the flow rate of unstimulated whole saliva (UWS) (rs 0.563, $p = 0.029$). No correlations with ESSDAI scores or other clinical parameters were found in this small study population.

Discussion

The main aim of this study was to gain insight in the formation of LELs by quantification of the numbers of B- and T-lymphocytes within striated ducts without hyperplasia and within different stages of LELs in the parotid and labial salivary gland of pSS patients, compared to salivary glands of non-SS sicca patients and non-sicca controls. We found that all analysed striated ducts with lymphocytes consistently harboured T-lymphocytes, scattered throughout the whole epithelium. In contrast, B-lymphocytes were exclusively seen in striated ducts without hyperplasia and in striated ducts with LELs of pSS patients, and were mostly located in areas of proliferating epithelium. B-lymphocytes were completely absent in the striated ducts of non-SS sicca and non-sicca controls. Absolute

Fig. 3. B-lymphocytes are exclusively present in striated ducts of pSS patients, and their absolute numbers increase with higher severity of LELs.



Numbers of B- and T-lymphocytes, as well as B/T-ratios were assessed in striated ducts without hyperplasia (SD w/o H) and striated ducts with hyperplasia (LELs) of pSS and control patients. LELs were only present in pSS patients. Striated ducts without hyperplasia of non-sicca controls (con), non-SS sicca patients (non-SS) and pSS patients (pSS) are listed on the left side of each graph. Striated ducts with LELs (stage 1-3) of pSS patients are listed on the right side of the graphs. Bars represent medians. GEE analyses were used to analyse differences in numbers of B-lymphocytes (A, B), T-lymphocytes (C, D) and B/T ratios (E, F) between SD w/o H of pSS and control patients (left side of graphs) and between the different LEL stages of pSS patients (right side of graphs) in both the parotid (A, C, E) and the labial salivary gland (B, D, F). Only significant differences are shown.

*** $p < 0.001$, ** $p = 0.001$, * $p < 0.05$.

numbers of B-lymphocytes increased with higher severity of LELs in both glands, leading to a significant increase in B/T-ratio over the stages of LELs. This was even more pronounced in the parotid gland, than in the labial gland. Our observation that in non-SS sicca patients (in this study defined as patients without anti-SSA antibodies and FS=0) and in non-sicca controls (all without periductal foci), the ductal epithelium only contains T-lymphocytes, indicates that these cells are a normal component of the salivary gland epithelium. On the other hand, B-lymphocytes were only present in a small proportion of striated ducts without hyperplasia and in all (parotid gland) or nearly all (labial glands) of the striated ducts with LELs of pSS patients. These B-lymphocytes are predominantly located at the sites where the epithelium is hyperplastic. These findings strongly argue that intraepithelial B-lymphocytes are involved in proliferation of the epithelial cells and the generation of LELs. Strong support for this notion also comes from studies of pSS patients treated with B-lymphocyte depletion therapy (rituximab). These studies showed that this therapy not only results in depletion of intraepithelial B-lymphocytes of the ducts but concomitantly also in downstaging of LELs (9). In this study, the possible contribution of hyperactive B-lymphocytes in salivary gland dysfunction was also reflected by positive correlations between B/T-ratios and severity of LELs with serological parameters and salivary secretion. However, our study population was quite small and correlations with clinical parameters should be further explored in a larger cohort of pSS patients.

Based upon the results of the current study, it seems plausible that influx of B-lymphocytes precedes the hyperplastic reaction of the epithelium, since 21% of striated ducts with lymphocytes, but without hyperplasia of pSS patients (but not of controls), already contain B-lymphocytes. A major chemokine produced by the ductal epithelium of pSS patients that can attract CXCR3-expressing B- and T-lymphocytes, is the inflammatory chemokine CXCL10, which is induced by IFN (10). Strongly

elevated levels of this chemokine are detected in saliva, tears and serum of pSS patients (11, 12). We have previously observed that the vast majority of B-lymphocytes within the LELs express FcRL4 (13). A high proportion of these intra-epithelial B-lymphocytes is proliferative in both the parotid (mean $20.5 \pm 7.7\%$) and labial gland (mean $15.4 \pm 8.2\%$), as revealed by Ki67 staining (13). Thus after the initial influx into the epithelium, these cells may proliferate locally. The reason for the proliferation of the B-lymphocytes (and possibly to some extent also intraepithelial T-lymphocytes) is not known. The epithelial cells in the inflamed salivary glands of the pSS patients produce a wide variety of cytokines, including BAFF and APRIL (14), which may support the intraepithelial proliferation of the B-lymphocytes. Verstappen *et al.* (15) revealed expression of IL-21 within the parotid ductal epithelium. This cytokine, important for B-lymphocyte activation and differentiation, is a signature cytokine for follicular helper T-lymphocytes (T_{FH}). This observation opens the possibility that T_{FH} play a role in the activation of intraepithelial B-lymphocytes.

Interestingly, also the MALT lymphomas in the parotid gland of pSS patients express FcRL4 and the neoplastic B-lymphocytes are characteristically associated with LELs. We therefore hypothesised that the neoplastic B-lymphocytes arise from this small subset of FcRL4-expressing B-lymphocytes (13). In line with the observation that MALT lymphomas preferentially develop in the parotid glands of pSS patients we observed in this study that B/T ratios are higher in striated ducts with LELs of parotid glands compared to labial glands. We speculate that after homing into the epithelium, the (FcRL4⁺) B-lymphocytes activate the ductal epithelial (stem) cells, after which they start to proliferate. How these B-lymphocytes exert their possible effects on the epithelium remains to be shown, but cytokines secreted by activated B-lymphocytes are possible candidates. It is already known that intestinal intraepithelial lymphocytes can secrete pro-inflammatory cytokines

like IL-2, IL-4 and IL-6, which can cause epithelial cell proliferation (16). Vice versa, epithelial stem cells derived from the parotid glands proliferate under the influence of pro-inflammatory cytokines in vitro (17). In RA, FcRL4⁺ B-lymphocytes have been found in synovial fluid, where they produce cytokines such as TNF α and RANKL (18). In salivary glands of pSS patients, FcRL4⁺ B-lymphocytes may possibly also secrete cytokines within the ductal epithelium and thereby stimulate proliferation of the epithelial cells [Verstappen *et al.* submitted for publication]. More research on the functional capabilities of the (FcRL4⁺) B-lymphocytes in pSS patients is needed, especially on the cytokines they produce.

In conclusion, LELs are a characteristic histopathological finding in salivary glands of pSS patients and there is a close interaction between lymphocytes and the ductal epithelial cells. Our results show an association between intra-epithelial B-lymphocytes and hyperplasia of the basal ductal epithelial cells and thereby the formation of LELs in salivary glands of pSS patients.

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