Serological Implications of Germinal Center-like Structures in Primary Sjögren's Syndrome

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ABSTRACT. ***Objective.*** To determine serological implications of germinal center (GC)-like structures in primary Sjögren's syndrome (pSS).

Methods. Retrospectively, minor salivary gland biopsies (n = 269) with focal lymphoid aggregates corresponding to focus score ≥ 1 were evaluated for the presence of GC-like morphology. Relevant clinical information was obtained from medical records.

Results. Of 269 patients, 169 fulfilled the American-European criteria for pSS. GC-like features were observed in 47/169 (28%) biopsies. In the majority of cases, GC-like lesions were confirmed by CD21-positive follicular dendritic cell networks. Mean inflammatory focus score was significantly higher in GC-positive compared to GC-negative samples ($p < 0.001$). GC-positive patients had lower mean salivary secretion ($p < 0.001$) and a higher frequency of patients with unstimulated salivary secretion ≤ 1.5 ml/15 min ($p < 0.01$). In addition, elevated titers of rheumatoid factor, serum anti-Ro/SSA and anti-La/SSB ($p < 0.05$), and IgG levels ≥ 15.3 g/l ($p < 0.05$) were more common in GC-positive compared to GC-negative. Enlarged salivary glands were observed in 46/163 (28%) patients, but could not be linked to either presence or absence of GC-like features.

Conclusion. Inflammatory infiltrates with GC-like morphology were observed in 28% of the investigated patients with pSS, and was particularly noted in patients with higher focus score. The observed serological aberrations in patients with ectopic GC-like structures in the minor salivary glands warrant further prospective studies. (First Release Sept 1 2007; J Rheumatol 2007;34:2044–9)

Key Indexing Terms:
FOCAL INFLAMMATION | GERMINAL CENTER FORMATION
LYMPHOID NEOGENESIS | SALIVARY GLAND | SJÖGREN'S SYNDROME

Sjögren's syndrome (SS) is an autoimmune chronic inflammatory disorder that predominantly affects the salivary and lacrimal glands, giving rise to symptoms such as oral and ocular dryness (xerostomia and keratoconjunctivitis sicca). Serum autoantibodies (Ro/SSA and La/SSB) may be detected in 50%–90% of patients[1,2]. Histopathologically, the disease is manifested by focal mononuclear cell infiltrates in the salivary and lacrimal glands. The infiltration is progressive and mainly consists of T lymphocytes, but also B lymphocytes and macrophages[3].

Ectopic follicles/germinal center (GC)-like structures have been detected in 20%–25% of patients with SS[4-7]. Similar features have also been described in affected tissue in other autoimmune diseases such as the synovium in rheumatoid arthritis (RA)[8,9], the thymus in myasthenia gravis[10], and the central nervous system in multiple sclerosis[11], during chronic *Helicobacter pylori* infections in gastric mucosa[12], in chronic inflammatory disorders of the liver[13], the thyroids in Hashimoto's disease[8], and in oral buccal mucosa related to amalgam fillings and lichenoid reactions[14,15].

Compared to the general population, patients with pSS are reported to have a 16 to 44-times increased risk of developing lymphoma, primarily extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue-type/non-Hodgkin's lymphoma[16,17]. A possible relationship between ectopic GC formation and malignant transformation has been suggested[16], and the higher risk of malignant transformation seems to be associated with purpura/skin vasculitis, low complement factor C3, low C4, CD4+ T lymphocytopenia, and a

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low CD4+/CD8+ T cell ratio[17,18]. Serum levels of soluble B cell-activating factor (sBAFF) are elevated in patients with SS[7,19,20] and non-Hodgkin's lymphoma[21], and serum sBAFF levels may correlate with autoantibody titers[20]. We have detected an association between local production and systemic autoantibody levels in patients with ectopic GC-like structures[6]. Although high focus scores and hypergammaglobulinemia were found to be more frequent in patients with ectopic GC, univariate analyses could not detect an association between ectopic GC-formation and BAFF or A-proliferation-inducing ligand (APRIL)[7].

The formation of ectopic GC-like structures in chronic inflammation is not a recent observation in SS or other autoimmune diseases, but the role and consequence of tertiary lymphoid structures in SS has not been thoroughly investigated. Our aim was to explore and determine if GC-like structures in primary SS (pSS) define a distinct serological phenotype. In a large sample of minor salivary gland biopsies, the occurrence and formation of ectopic GC-like structures was determined by histopathological evaluation and related to serological aberrations and clinical findings.

MATERIALS AND METHODS

Patients and tissue samples. The consecutive materials of 269 patients evaluated for SS at Haukeland University Hospital between 1989 and 2005 were investigated. Among the 269 patients, 169 fulfilled the revised American-European criteria for pSS[22] and 35 patients fulfilled secondary SS (sSS) criteria with additional rheumatic diseases such as RA[23] (n = 28), systemic lupus erythematosus[24] (n = 3), ankylosing spondylitis (n = 1), CREST syndrome (calcinosis, Raynaud's, esophageal dysmotility, sclerodactyly, telangiectasias; n = 1), polymyositis (n = 1), and scleroderma (n = 1). For the remaining 65 individuals, medical records were not accessible and it was not possible to ascertain either pSS or sSS. Hematoxylin and eosin (H&E)-stained paraffin-embedded minor salivary gland tissue sections (n = 269) were morphologically screened for the presence of ectopic GC-like structures. Inflammation had previously been determined by focus score, i.e., the number of inflammatory cell foci containing at least 50 mononuclear cells per 4 mm^2. All sections evaluated had SS-like focal inflammation (Figure 1A) with a focus score ≥ 1. A GC-like structure was defined as a well circumscribed chronic inflammatory cell infiltrate consisting of at least 50 mononuclear cells, presenting with a densely packed dark zone and a light zone, within otherwise normal salivary gland epithelium (Figure 1B), in comparison to conventional focal infiltrates where such lymphoid organization was not observed (Figure 1A). Focal infiltrates and GC-like structures may be present within the same minor salivary gland[7]. In such cases the section was characterized as GC-positive.

Our study was approved by the Committee of Ethics at the University of Bergen (145/96-44.96 and 242.06).

Immunohistochemistry. Ectopic GC-like features were further investigated in 60 randomly selected biopsies. Paraffin-embedded salivary gland tissue was cut (4–6 mm sections) with a microtome (Leica Instruments GmbH, Nussloch, Germany) onto SuperFrost® Plus microscope slides (Menzel GmbH & Co. KG, Braunschweig, Germany). Following deparaffinization and rehydration, heat-induced epitope retrieval (HIER) was performed in a microwave oven using citrate buffer, pH 6.0 (S1699). Endogenous peroxidase activity was quenched by Peroxidase Block. Sections were incubated with primary antibody CD21 (1:50, monoclonal mouse anti-human IgG1 kappa, clone 1FB) for 60 min and thereafter with horseradish peroxidase-conjugated anti-mouse EnVision+ for 30 min. Optimal dilution of the primary antibody was determined after serial titrations. Diaminobenzidine (DAB+) was used as chromogen.

Sections were counterstained with hematoxylin, dehydrated, and mounted with a non-aqueous mounting medium (Eukitt) prior to analysis. Between each step, sections were carefully washed with TBS, pH 7.6, for 2 × 5 min. Except for the HIER, all steps were performed at room temperature. Unless otherwise indicated, all reagents were purchased from Dako A/S, Glostrup, Denmark. All tissue sections were analyzed in duplicate.

Evaluation of staining. Sections were evaluated using a light microscope (Leica DMLB) with a 20× objective. The staining pattern of CD21 within chronic inflammatory cell infiltrates, i.e., accumulations of 50 or more mononuclear cells surrounded by otherwise normal salivary gland tissue, was noted. Roughly, inflammatory cell infiltrates could be divided into ones that did not exhibit any immunohistochemical staining, or where a few scattered cells with membrane-bound staining and initiation of network-like morphology could be observed, and lastly, ones where positive cells with membrane-bound staining was observed on interacting cells, forming small well circumscribed follicles and/or a GC within the infiltrate. Samples where the focal infiltrates were negative for CD21 and samples exhibiting only initiating network-like staining of CD21-positive cells were considered GC-negative, and the remainder were GC-positive.

Clinical and immunological data. Following the histopathologic evaluation, patients with pSS were investigated further. Medical records were obtained for all patients, with focus on levels of rheumatoid factor (RF), antinuclear antibodies (ANA) and autoantibodies to the Ro/SSA and La/SSB proteins, levels of immunoglobulins IgG, IgA and IgM and general markers of inflammation such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), complement factor C3 and C4 levels, and unstimulated whole saliva per 15 min. All values were obtained as close to the time of the minor salivary gland biopsy as possible.

RF detection was performed by the Waaler reaction with titers < 128 considered normal. ANA titers were considered normal when < 128. Anti-Ro/SSA and anti-La/SSB were measured by standard commercial ELISA as positive or negative. Immunoglobulin levels were measured by nephelometry (normal values: IgG 6.0–15.3 g/l, IgA 1.0–4.1 g/l, IgM 0.5–2.5 g/l). Normal range for ESR in women age < 50 years 1–20 mm/h, women > 50 years 1–30 mm/h, men < 50 years 1–15 mm/h, and men > 50 years 1–20 mm/h. CRP was detected by radial nephelometry and considered normal when < 10 mg/l. Complement C3 levels were considered low when ≤ 0.83 g/l, and C4 levels when ≤ 0.18 g/l. Unstimulated whole salivary secretion was considered decreased when ≤ 1.5 ml/15 min. Findings are summarized in Table 1.

Statistical analyses. Data were frequently not normally distributed. We used the Mann-Whitney test to study differences between groups and Spearman correlation for relationships between variables. Chi-square analysis was employed for categorical data. All statistical analyses were performed by use of SPSS 13.0.

RESULTS

Characteristics of the study population. The material investigated (n = 169) consisted of 160 women and 9 men, all fulfilling the revised criteria for the diagnosis of pSS[22]. Clinical and laboratory characteristics are given in Table 1. Focus score was significantly higher in Ro/SSA-positive ($p < 0.05$) and La/SSB-positive patients ($p < 0.001$) and correlated positively with serum IgG ($r = 0.359$, $p < 0.001$). Salivary secretion correlated negatively with serum IgA levels ($r = –0.248$, $p < 0.01$). A negative association was found between C4 levels and disease progression as assessed by increased focus score ($r = –0.293$, $p < 0.05$). Swollen or enlarged major salivary glands were observed in 28% of the pSS population investigated.

GC-like structures in pSS and sSS. In the initial screening of salivary gland tissue from patients with pSS and sSS, focal sialadenitis (Figure 1A) was present in all 269 biopsies. In

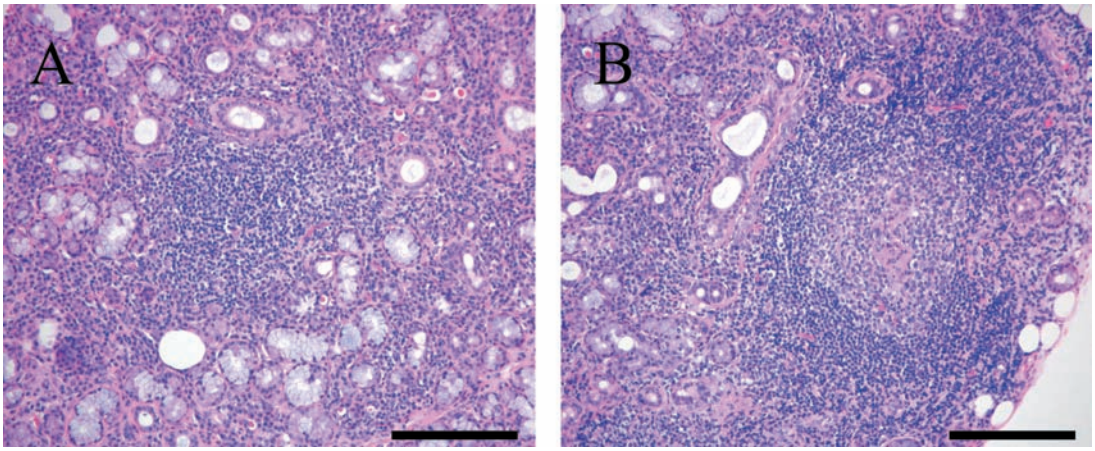

Figure 1. Germinal centers were identified by morphology in 47/169 H&E biopsies. The figure illustrates a conventional focal infiltrate (A) and an infiltrate with GC-like morphology (B). Both types of inflammation are surrounded by otherwise normal-appearing salivary gland tissue. In the H&E sections, GC-like structures were detected by the appearance of a well circumscribed chronic inflammatory cell infiltrate, a densely packed dark zone, and a light zone, as compared to the focal infiltrate, where such lymphoid organization was not observed. Bar = 1 mm.

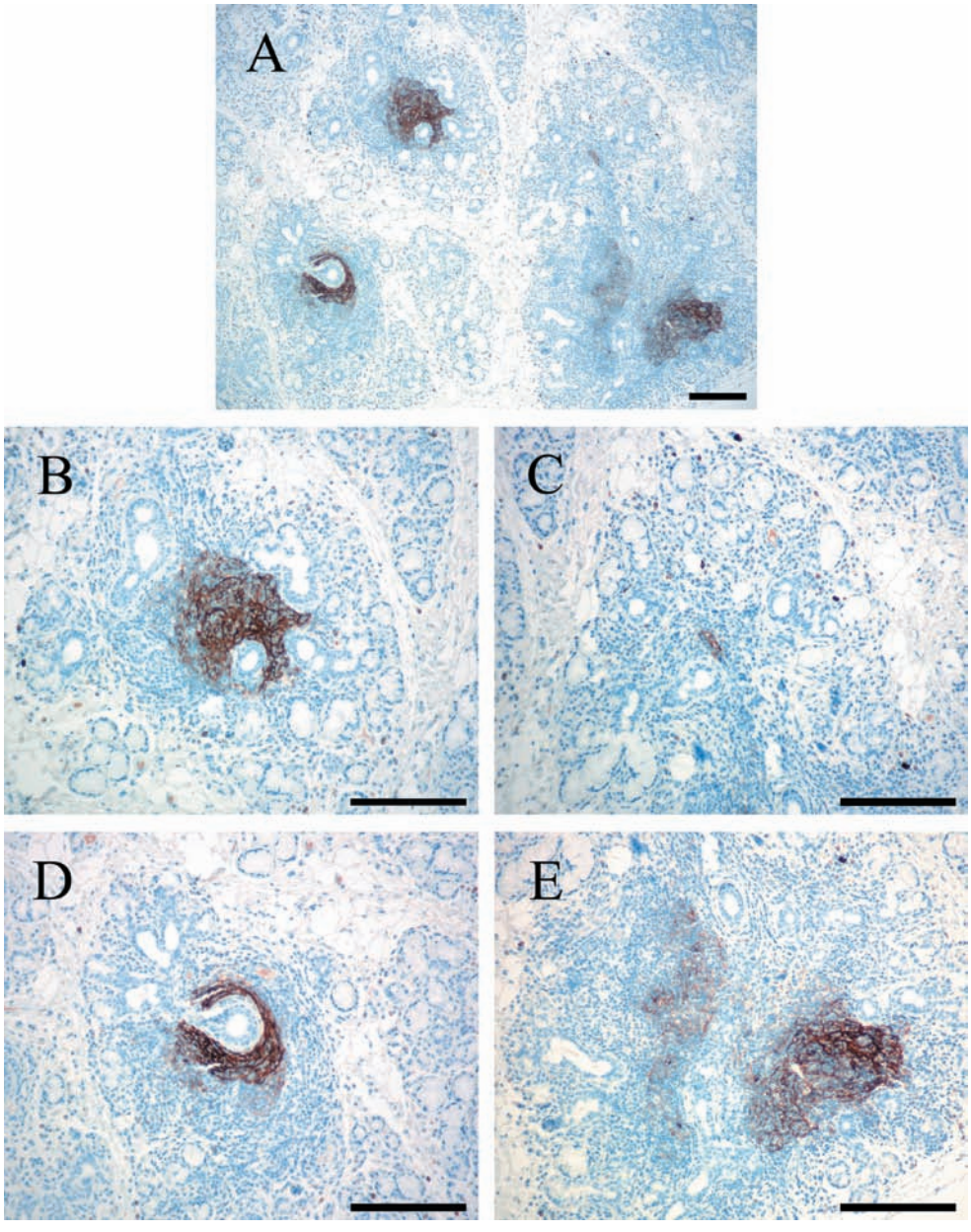

Figure 2. In 60 randomly selected biopsies, GC-like features and focal inflammation were investigated by immunohistochemistry. In the routine H&E tissue sections, GC-like morphology had been observed in 11/60 (18%) biopsies. The GC-like inflammation was confirmed by GC-marker CD21, which is present on follicular dendritic cells and B cells. As illustrated in the overview (A), several staining patterns were sometimes noted within the same biopsy. The various staining patterns are shown in detail (B to E). CD21+ follicles (B and E right) were observed in 9/11 tissue samples previously characterized as GC-positive. In the remaining 2 samples, a few CD21+ cells (C) and network-like CD21+ staining indicating an early stage of ectopic follicle formation (D and E left) were observed. Bar = 1 mm.

Table 1. Clinical information from all patients that fulfilled the American-European classification criteria for pSS[22]. Fractions indicate the number of patients with the observed phenomenon, of the total numbers of patients where this had been investigated. For age, complement factor C3 and C4, focus score, and unstimulated whole salivary secretion, results are presented as median ± 25th–75th percentile.

Characteristic	
No.	169
Age, yrs	54 ± 45–62
Rheumatoid factor (Waaler titer ≥ 128)	24/161
Antinuclear antibodies (titer ≥ 128)	76/162
Ro/SSA (positive/negative)	70/163
La/SSB (positive/negative)	41/161
IgG (≥ 15.3 g/l)	70/160
IgA (≥ 4.1 g/l)	20/159
IgM (≥ 2.5 g/l)	23/159
Erythrocyte sedimentation rate*	42/123
C-reactive protein (≥ 10 mg/l)	13/89
Complement factor C3 (g/l)	1.04 ± 0.88–128
C3 (≤ 0.83 g/l)	12/50
Complement factor C4 (g/l)	0.20 ± 0.15–0.26
C4 (≤ 0.18 g/l)	17/50
Focus score	2.0 ± 1.0–4.0
Unstimulated whole salivary secretion per 15 min	1.20 ± 0.40–2.38
Salivary secretion ≤ 1.5 ml/15 min	76/121
Swollen salivary glands	46/163

* ESR normal range in women < 50 yrs 1–20 mm/h, women > 50 yrs 1–30 mm/h; men < 50 yrs 1–15 mm/h, men > 50 yrs 1–20 mm/h.

addition, GC-like morphology (GC-positive; Figure 1B) was detected in 59/269 (22%) biopsies. In the salivary gland tissue from patients fulfilling pSS criteria[22] this made up 47/169 (28%) biopsies. Five of the 35 (14%) patients with sSS and 6 of the 65 (9%) individuals grouped as focal sialadenitis had morphologic features of ectopic GC-like structures in the minor salivary glands (Table 2).

Immunohistochemistry and GC-like structures. Tissue sections from randomly selected patients with pSS (n = 60) were further investigated by immunohistochemistry. In the preliminary screening on H&E routine sections, GC-like morphology (GC-positive) had been observed in 11 of these 60 (18%) randomly selected biopsies. Using immunohistochemistry, several different staining patterns could be observed in the minor salivary gland tissue sections (Figure 2A). Well circumscribed CD21-positive follicles (Figure 2B and 2E) were observed in 9 of the 11 (82%) GC-positive biopsies. In the remaining 2, one showed scattered CD21-positive cells with membrane-bound staining (Figure 2C) and initiating network-like morphology (Figure 2D and 2E left), while the last one lacked immunohistochemical staining in the focal infiltrate (not shown).

Table 2. GC-like structures were detected in 47/169 (28%) of the minor salivary glands from patients with primary Sjögren's syndrome (pSS), in 5/35 (14%) secondary SS (sSS), and in 6/65 (9%) of the minor salivary glands characterized as focal sialadenitis (FS). Focal sialadenitis indicates the presence of SS-like focal inflammation, but lack of sufficient clinical information to determine a pSS/sSS diagnosis.

	pSS	sSS	FS	Total
GC-positive	47	5	6	58
GC-negative	122	30	59	211
Total	169	35	65	269

GC-like structures coincide with serological aberrations in pSS. When dividing the pSS patient material according to morphologic GC-like changes in the minor salivary glands, focus scores were significantly higher in the GC-positive samples (Table 3) and were negatively correlated with salivary secretion ($r = –0.353$, $p < 0.05$), whereas in the GC-negative samples, focus scores correlated positively with salivary secretions ($r = 0.250$, $p < 0.05$). GC-positive patients had higher frequency of pathologic Ro/SSA and La/SSB levels, higher IgG levels, and reduced salivary secretion compared to the GC-negative patients (Table 3). In the GC-negative patients, a positive correlation was detected between focus score and IgG ($r = 0.353$, $p < 0.001$) and IgM ($r = 0.188$, $p < 0.05$). Negative correlations were detected between C3 levels and focus score ($r = –0.362$, $p < 0.05$) and salivary secretion ($r = –0.465$, $p = 0.01$; data not shown). Reduced C4 levels (≤

Table 3. Serological, clinical, and histopathological findings in patients with pSS investigated for ectopic GC-like morphology (n = 169). Findings in patients with morphologic GC-like structures (GC-positive) are compared to patients with focal infiltrates lacking such lymphoid organization (GC-negative). Fractions indicate number of patients with the observed phenomenon, of the total number of patients where this had been investigated. For age, complement factor C3 and C4, focus score, and unstimulated whole salivary secretion, results are presented as median ± 25th–75th percentile.

Characteristic	GC-positive	GC-negative
No. of patients	47	122
Age, yrs	52 ± 39–59*	56 ± 48.5–63.3
RF (≥ 128)	10/44	14/117
ANA (≥ 128)	22/45	54/117
Ro/SSA (positive/negative)	25/45*	45/118
La/SSB (positive/negative)	17/44*	24/117
IgG (≥ 15.3 g/l)	27/45*	43/115
IgA (≥ 4.1 g/l)	4/45	16/114
IgM (≥ 2.5 g/l)	7/45	16/114
ESR†	13/38	29/85
CRP (≥ 10 mg/l)	4/30	9/59
Complement factor C3 (g/l)	1.20 ± 0.81–1.34	1.07 ± 0.86–1.36
C3 ≤ 0.83 g/l	5/19	7/31
Complement factor C4 (g/l)	0.20 ± 0.18–0.25	0.21 ± 0.16–0.27
C4 ≤ 0.18 g/l	6/19	11/31
Unstimulated whole salivary secretion	0.50 ± 0.10–1.30***	1.30 ± 0.50–3.00
Salivary secretion ≤ 1.5 ml/15 min	30/37**	46/84
Focus score	3 ± 2–4***	2 ± 1–3
Swollen salivary glands	14/45	32/118

† ESR normal range in women < 50 yrs 1–20 mm/h, women > 50 yrs 1–30 mm/h; men < 50 yrs 1–15 mm/h, men > 50 yrs 1–20 mm/h. Statistically significant differences: * $p < 0.05$; ** $p < 0.01$; *** $p \leq 0.001$.

0.18 g/l) were observed in 35.5% of GC-negative samples compared to 31.5% in GC-positives. Serum levels of IgA also correlated negatively with secretion of saliva in the GC-negatives ($r = -0.233$, $p < 0.05$). Swollen salivary glands were observed in 31.1% of the GC-positive and in 27.1% of the GC-negative samples, a nonsignificant difference, and could not be significantly linked to any of the other serological aberrations in the investigated material.

DISCUSSION

We and others have described ectopic GC formation in the salivary glands of patients with pSS and sSS[4,6,7,25]. In accordance with previous findings in small cohorts of patients with SS, patients with ectopic GC-like structures in this material presented with higher levels of serum autoantibodies[6], IgG, focus score (progressive disease), and elevated titers of RF[7]. The significance of ectopic GC-like structures and their interactions with the surrounding stroma is not clear. However, the presence of antigen-presenting cells[7,26] and local autoantibody production suggest ongoing antigen presentation and immune activation.

Several aspects of the B cell components in chronic inflammation have been described in SS[1,27,28]. BAFF-expressing cells are considered important in the pathogenesis of pSS and have been linked to attenuated apoptosis[7,19,20,29,30]. We have reported membrane-bound BAFF staining on scattered infiltrating cells that was comparable in GC and focal infiltrates[7]. Serum levels of sBAFF were increased in pSS, and although similar in GC-positive and GC-negative patients, sBAFF levels correlated with increased focus score in the GC-positive patients. No significant correlations were detected in the GC-negative patients[7]. However, the response of infiltrating B cells to BAFF may be modified by various cytokines[31] and this may differ in GC-positive and GC-negative patients. B cell dysfunction[32] and disturbances of B cell maturation[27,33] have been demonstrated in patients with SS, implying that overstimulation and neglected selection may take place in induced GC in the affected glands.

Development of lymphoma has been associated with BAFF[21] and ectopic GC formation, and increased risk for development of extraglandular disease has been suggested[16]. Low levels of complement factors C3 and C4 and swollen salivary glands have been described as risk factors for development of extraglandular disease such as lymphoma[17]. In our cohort of patients with pSS (n = 169), C4 levels correlated negatively with glandular disease severity as assessed by a higher focus score, i.e., patients with high focus score tended to have reduced C4 levels ($\leq$ 0.18 g/l), indicating increased use of complement. Although low levels of C3 ($\leq$ 0.83 g/l) were slightly more commonly observed in the GC-positive patients, who also had higher focus scores compared to GC-negative patients, a significant negative correlation between serum C3 levels and focus score was only observed in the GC-negative patients. Unfortunately, records concerning C3/C4 levels were only available from 50 patients in the cohort, and although interesting, results may not be considered representative for the general pSS population. Concerning swollen salivary glands, no conclusive observations concerning serological aberrations and the presence/absence of GC-like features could be made in this cohort.

In addition to morphologic appearance, GC-like structures in SS have been characterized by CD3+ T and CD20+ B cell staining, presence of Ki-67+ proliferating cells, CD35+ follicular dendritic cell (FDC) networks, and CD31+ high endothelial venules[6]. In the majority of cases investigated in our study, GC-like structures in minor salivary glands were confirmed by CD21+ FDC networks. The presence of proliferating cells, FDC networks, and an IgD+ mantle zone, maintained over time, were previously suggested to identify ectopic GC-like structures in RA synovia[34]. Albeit at significantly lower levels, we have detected proliferating cells in focal infiltrates as well as ectopic GC-like structures[7], and it is speculated whether ectopic GC-like structures occur in all patients with SS at some timepoint. Indeed, SS is a progressive disorder[3] but because of ethical considerations, repeated biopsies are not routinely performed in SS, unless malignant lymphoproliferative disease is suspected.

GC-positive patients presented with higher mean focus scores and elevated titers of RF, serum autoantibodies, and IgG levels. In view of this, the occurrence of GC-like features in the salivary glands of patients with pSS may have several implications, both by promoting loss of salivary gland tissue and by acting as a tertiary lymphoid organ. Whether immune responses within the salivary glands are restricted to antigens present in the local environment, or whether antigen trapping and enrichment could also become relevant for antigens not primarily involved in the disease process, is not clear. Defective lymphatic drainage of the inflamed tissue and enduring local antigenic stimulation have been suggested to be crucial triggers in the cascade of events leading to lymphoid neogenesis[35]. Lymphoid organization in peripheral tissues imposes a possible risk for immune recognition and breakdown of self-tolerance, or may be a mere but undesirable consequence of continuous production of cytokines and other inflammatory mediators by salivary gland stromal cells, or a result of chronic local stress. Nevertheless, autoimmune diseases are the result of a multistep process where genetic and environmental factors interact over a long period of time. The presence of lymphoid organization within the salivary glands of patients with pSS may be an important step in the timing and consolidation of the autoimmune response. Although our results could not be linked to known risk factors such as swollen salivary glands and lower complement factor C3/C4 levels, they indicate a certain serological phenotype for SS patients with ectopic lymphoid organization, and warrant further prospective studies on lymphoid neogenesis in relation to disease progression and possible treatment options.

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