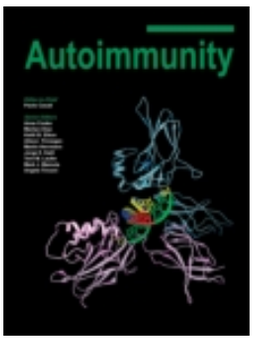

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Low number of memory B cells in the salivary glands of patients with primary Sjögren's syndrome

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Abstract
We have previously shown that patients with primary Sjögren's Syndrome (pSS) show a significant reduction of autoantigen specific $CD27^+$ memory B cells and an abnormally elevated level of autoantibody producing plasma cells in peripheral blood (PB) compared to controls. Because both memory B cells and plasma cells have been detected in salivary glands (SG) of pSS patients, we aimed to study the B cell pattern in SG biopsies. Double immunohistochemical staining of CD20 and CD27 was carried out on paraffin-embedded SG tissue from 10 pSS patients to distinguish $CD20^+/CD27^+$ memory B cells, and identify the $CD20^+$ glandular B cell zones (BCZ). Given that plasma blasts and plasma cells are $CD27^{++}$ and $CD20^-$, additional CD138 single staining of serial sections allowed the distinction of $CD27^{++}/CD138^-$ plasma blasts located within the BCZ from $CD27^{++}/CD138^+$ plasma cells that were found mostly on the periphery of the BCZ and also observed interstitially. Both BCZ and the memory B cell populations were then quantified. Contrary to what has been reported earlier through immunoflourescent staining of memory B cells in SG tissue, we have shown that there is a low number of memory B cells located within the glandular BCZ. Plasma blasts and plasma cells, however, were more abundant in the SG. Together our findings suggest that these low numbers of memory B cells in both PB and SG of pSS patients may be the result of activation of these cells into plasma cells at the site of inflammation.

Keywords: *Autoimmunity, B cell zones, memory B cells, plasma cells, salivary glands, Sjögren's syndrome*

Abbreviations: *AECC: American-European consensus group criteria, ANA: antinuclear antibodies, AP: Alkaline phosphatase, BCZ: B cell zones, DAB: diaminobenzidine, FS: focus score, GC: germinal centre, H&E: haematoxylin and eosin, HIER: heat-induced epitope retrieval, HRP: horse radish peroxidase, LPR: liquid permanent red, IF: immunoflourescent, IHC: Immunohistochemical, NG: normal glands, PB: peripheral blood, pSS: primary Sjögren's syndrome, RF: rheumatoid factor, RT: room temperature, SS: Sjögren's syndrome, SG: salivary glands and TBS: tris-buffered saline, TBST: TBS containing 0.1% Tween*

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterised by chronic inflammation that is manifested by mononuclear cell infiltration of exocrine glands, particularly lachrymal and salivary glands (SG) [1,2]. This results in impaired tear and saliva production, which in turn contribute to the clinical symptoms of dry eyes and dry mouth [3]. Another characteristic feature of pSS is high serum titres of Ro/SS-A, La/SS-B antigens, and rheumatoid factor (RF) autoantibodies [4–8]. This in turn results in a state of hypergammaglobulinaemia where pSS patients generally show an increase of naïve B cells, and consequently an elevated level of antibody secreting plasma cells [9]. The enhanced B cell differentiation in Sjögren's syndrome (SS) also results in a depressed percentage of circulating memory B cells and elevated levels of soluble CD27 that also correlate with serum IgG concentrations [10,11]. This further suggests an abnormal memory B cell compartment in pSS [12].

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Memory B cells are distinguished by their expression of surface marker CD27 [13,14]. However, plasma blasts, plasma cells and long-lived plasma cells are also $CD27^{++}$ [14–17]. In addition, plasma cells and long-lived plasma cells both express CD138, a feature that distinguishes them from plasma blasts [18–20]. Still, plasma blasts and plasma cells are CD20-negative, and naïve and memory B cells express CD20 [12,21,22]. This may explain why present therapeutic interventions in SS that depend on the targeting and elimination of CD20-positive cells lead to reduced numbers of B cells in the patients yet unchanged serum levels of autoantibodies, since the CD20-negative autoantibody producing plasma cells, which can be the long-lived subset, remain unaffected by treatment [23–25]. Still, the anti-CD20 antibody Rituximab® is the most successful antibody so far developed for therapeutic purposes, and has proven to be effective and well tolerated by those SS patients that were also diagnosed with non-Hodgkin's lymphoma [26–30].

The focal infiltrates in the SG tissue of SS patients consist of T cells, B cells, plasma cells, macrophages, follicular dendritic cells, dendritic cells, and plasmacytoid dendritic cells [31–34]. Both anti-Ro/SSA and anti-La/SSB autoantibody-producing cells have also been detected in the SG of SS patients [35–38]. These infiltrating cells can result in the formation of germinal centres (GC) in approximately 25% of pSS patients, which could be the result of antigen-dependent immune responses in the secondary lymphoid tissue [39–41].

Nonetheless, B cells have been found to make up 20% of the infiltrating cell population in exocrine glands [42]. A great portion of these B cells have been shown to include both plasma cells and long-lived plasma cells in an activated stage [18]. Furthermore, memory B cells have also previously been shown to accumulate in the SG of patients with SS [43,44]. This was detected by immunoflourescent (IF) staining of parotid tissue obtained from 1 SS patient that was also diagnosed with lymphoma [43].

In the present study we aimed to further explore the B cell pattern in the SG of 10 patients diagnosed with pSS. By applying double immunohistochemical (IHC) labeling of CD20 and CD27 on paraffin-embedded tissue sections of SG, followed by single staining with CD138, we identified the glandular B cell zones (BCZ) and gained insight on the morphology and distribution of the different subtypes of B cells present within the gland. Given that the peripheral blood (PB) of these subjects has been analysed in our previous study where the number of $CD27^{+}$ autoantigen specific memory B cells was found to be low [45], we can further examine the impact this could have on the number of memory B cells infiltrating the SG tissue of these same individuals. Together, our findings could give further insight into the potential efficiency of current therapeutic strategies that rely on depletion of CD20-positive cells in SS patients.

Methods

Study population

Lower labial minor SG biopsies attained from 10 patients that were diagnosed with pSS have been included in this study, 9 of which fulfilled the American-European consensus group criteria (AECC) for pSS [46]. The different biopsies had been performed between the years 1992 and 2009 at the Department of Otolaryngology/Head and Neck Surgery at Haukeland University Hospital in Bergen, Norway. These formalin fixed, paraffin embedded minor SG tissue sections were stained with haematoxylin and eosin (H&E) and evaluated by an oral pathologist in order to determine their focus score (FS). This is defined as the number of mononuclear cell infiltrates with >50 mononuclear cells per $4\,mm^2$.

At the time, our study population included varying FS from 1 to 4. However, focus scoring is a semi-quantitative method, and these FS values may differ depending on how deep in the gland the sections were taken. For this reason, new H&E staining was performed for all 10 patients and their FS was re-evaluated to eliminate potential discrepancies. The present FS range was found to be from 1 to 3.

Furthermore, these H&E stained sections were also screened for the presence of GC like structures. Individuals that were evaluated for pSS, but did not fulfil the AECC criteria or had normal glands (NG) with no inflammation in their SG tissue served as non-pSS tissue controls. Both instances subsequently resulted in a FS value of zero.

Medical records and clinical data were obtained from patients' charts at the Department of Rheumatology, Haukeland University Hospital. This provided information collected during routine laboratory assessments such as RF detection, antinuclear antibodies (ANA), anti-Ro/SSA and anti-La/SSB. Moreover, PB and plasma, attained in the year 2010, from these 10 patients have also been examined in a previous study [45]. This provided new insight into Ro- and La-specific memory B cell pattern and function in relation to the progression of the disease in these individuals. In addition, a re-assessment of the autoantibody production in these subjects was also made feasible and preformed against each of Ro52, Ro60 and La48 autoantigens. The clinical and laboratory characteristics of the pSS patients included in this study are presented in Table I. All studied subjects gave their informed consent, and the Committee of Ethics at the University of Bergen approved the study.

Table I. Medical and experimental characteristics of the pSS patients included in the study.

Patient no.	ANA*	Ro52† (μg/ml)	Ro60 (μg/ml)	La48 (μg/ml)	RF titer	IgG (g/L)	IgA (g/L)	IgM (g/L)	‡%Ro60 of total IgG + Memory B cells in PB	‡%Ro52 of total IgG + Memory B cells in PB	‡%La48 of total IgG + Memory B cells in PB	Focus¶ score	Focus§ score (re-evaluated)	GC +/−	Salivary‖ secretion	Schirmer's test
pSS-138	−	00.0	00.0	00.0	+	7.62	3.78	0.47	2	7	0	4	2	+	NT	NT
pSS-141	+	5.83	16.0	19.4	+	21.4	4.38	3.10	0	11	0	3	3	−	1.00	+
pSS-144	+	00.0	00.0	00.0	−	6.58	1.08	0.85	3	5	1	2	0	−	12.0	−
pSS-147	+	0.29	00.0	3.01	−	14.4	6.59	0.28	1	0	1	1	1	−	−	+
pSS-149	+	00.0	00.0	00.0	+	9.08	1.85	1.18	0	1	3	4	1	−	0.20	NT
pSS-152	+	3.23	00.0	00.0	−	7.89	2.21	1.00	1	1	0	0	0	−	1.40	+
pSS-158	+	5.81	16.3	22.0	−	39.4	2.39	1.16	1	6	0	0	3	−	NT	+
pSS-160	+	00.0	00.0	00.0	+	11.1	1.82	1.00	1	1	1	2	0	−	0.50	−
pSS-163	+	00.0	00.0	00.0	−	15.7	2.17	0.63	0	1	0	1	2	−	0.00	−
pSS-165	−	00.0	00.0	0.58	−	8.49	1.94	0.59	1	0	0	1	1	−	0.60	+

ANA = antinuclear antibodies; RF = rheumatoid factor; GC = germinal centre; NT = not tested.
1) Aqrawi, L. A., K. Skarstein, G. Bredholt, J. G. Brun, and K. A. Brokstad. 2012. Autoantigen-specific memory B cells in primary Sjogren's syndrome. *Scand. J. Immunol.* 75(1): 61–68; ⋆ Values are attained by ELISA ranging at 0–9.99; †Autoantibody production was assessed by ELISA previously and presented in μg/ml for each Ro52, Ro60 and La48[1]; ‡Autoantigen specific memory B cells measured by ELISPOT previously and presented as a percentage of IgG + memory B cells[1]; ¶ Values are the number of focal infiltrates/4 mm^2 area containing > 50 mononuclear cells; §To eliminate discrepancies, new H&E staining was performed for all ten patients and their FS was re-evaluated; ‖ Values are in ml/15 min (unstimulated flow); normal flow > 1.5 ml/15 min.

Primary antibodies

The following primary anti-human antibodies were used in this study: mouse monoclonal CD138 (1:200) (clone MI15, Dako A/S, Glostrup, Denmark), mouse monoclonal CD27 (1:10) (clone 137B4, Nordic BioSite, Täby, Sweden), mouse monoclonal CD20 (1:3000) (clone L26, Dako A/S, Glostrup, Denmark), mouse monoclonal CD19 (1:50) (clone LE-CD19, Dako A/S, Glostrup, Denmark).

Immunohistochemistry

Single-staining. A Leica serial microtome (Leica Instruments GmbH, Nussloch, Germany) was used to cut paraffin embedded, formalin fixed minor SG tissue (4–6 μM thick). The sections were placed on SuperFrost® Plus microscope slides and incubated overnight in a heat cabinet at 56°C. This was followed by deparaffinisation in xylene, and rehydration through a graded ethanol series (100%, 96%, 70%) and distilled water. The sections were then subjected to heat-induced epitope retrieval (HIER) with citrate buffer (Target Retrieval Solution, pH 9.0, S2375, Dako, Glostrup, Denmark), and endogenous peroxidase activity was blocked with 0.3% peroxidase (K4007, Dako, Carpinteria, CA, USA) for 5 min.

Furthermore, the sections were incubated with primary antibody (CD138), made up in antibody diluent (S0809, Dako, Carpinteria, CA, USA), for 60 min. This was followed by incubation with horseradish peroxidase (HRP)- conjugated anti-mouse En Vision secondary antibody (K4007, Dako, Carpinteria, CA, USA) for 30 min. Thereafter, the sections were incubated for 10 min with diaminobenzidine (DAB), which was used as chromogen for development (K4007, Dako, Carpinteria, CA, USA). All incubations were performed at room temperature (RT), and Tris-buffer saline (TBS) containing 0.1% Tween was used as washing buffer (pH 7.6) between every step for 10 min. Finally, the sections were counterstained with Haematoxylin (S3301, Dako) for 4 min, dehydrated and mounted in Eukitt (O. Kindler GmbH & Co, Freiburg, Germany).

Double-staining. Expression of CD27 and CD20 was detected by double-staining. The sections were pre-treated as previously described and incubated with Dual Endogenous Enzyme Block (K5361, Dako, Glostrup, Denmark) for 10 min in order to block endogenous peroxidase and alkaline phospatase (AP). The first primary antibody (CD27) was then added and the sections were incubated for 60 min at RT. This was followed by the procedure described in the single-staining. After the development of the first primary antibody with DAB, the sections were washed with

water for 5 min, with TBST for 10 min, and then treated with Doublestain Block (K5361, Dako, Glostrup, Denmark) for 3 min.

Thereafter, the sections were incubated with the second primary antibody (CD20) for 10 min at RT, then placed at 4°C overnight, and again thawed for another 10 min at RT the following day. Binding of the second primary antibody was detected by the AP polymer and Permanent Red (PR, K5361, Dako, Glostrup, Denmark) incubation for 10 min. Similar to the single-staining method described previously, the sections were washed with TBST for 10 min between every step, and consecutively counterstained with haematoxylin, dehydrated, and mounted in Eukitt.

Evaluation of staining

The minor SG sections were studied using a light microscope (Leica, DMLB, Leica Microsystems Wetzlar, Wetzlar) by three investigators. Both mononuclear cells in focal infiltrates and those located interstitially i.e., in close proximity to the acinar or ductal epithelium were analysed.

The single-positive BCZ (CD20) and double-positive memory B cells (CD20/CD27) were counted using a grid and a 10X, 40X or 63X objective. Cells were considered positive when 50% or more of the cell membrane was positively stained. All the glandular tissue from each patient was counted. The total area of the minor SG section was also measured for each of the patients. Together, this allowed the presentation of the data as number of BCZ- and double-positive cells per 10 mm^2 of SG tissue.

Statistical analysis

Statistical significance was evaluated by the Student's *t*-test and presented as mean. Differences were considered significant when $p \leq 0.05$. In addition, the Pearson correlation test was used to examine the association between the different parameters.

Results

Study group

The 10 pSS patients used in this study were divided into 4 groups according to the degree of inflammation in their SG tissue. This was done by relying on their recently re-evaluated FS values. One group consisted of patients with FS = 0 that served as non-pSS tissue controls with normal SG morphology, since they exhibited little to no inflammation in their SG tissue. The 3 other groups included patients with FS = 1, FS = 2 and FS = 3, respectively.

When studying the morphology of the different SG sections, we detected GC-like structure (GC^+) in the SG of 1 patient, namely pSS-138, which had an FS of 2. This is consistent with what has been observed previously, explaining how GC^+ structures are more likely to occur in instances of increased inflammation and infiltrations, where the FS is ≥ 2 [18,35,40]. Interestingly, this GC^+ patient was negative for both ANA and autoantibodies (Ro52, Ro60 and La48). Contrary to this, 8 patients were ANA-positive, including the control group.

Patients in the FS = 3 group exhibited the highest values for Ro52, Ro60 and La48 autoantibody production. This resulted in an average of 5.82 μg/ml, 16.15 μg/ml, and 20.7 μg/ml for Ro52, Ro60 and La48, respectively. A similar pattern was observed for IgG and IgM measurements, where the FS = 3 group had a mean of 30.4 g/L and 2.13 g/L of IgG and IgM, in that order. On the other hand, no autoantibody production could be detected in patients with FS = 0, except in the case of pSS-152 that was to a lesser degree positive for Ro52 (3.23 μg/ml).

The percentage of IgG^+ memory B cells that are specific for Ro52, Ro60 and La48 was generally low in all 10 patients, ranging from 0 to 11%. Nonetheless, the highest percent values measured (6% and 11%) were for Ro52-specific IgG^+ memory B cells, and consequently observed in the FS = 3 group. Also, patients with no serum positivity of autoantibodies against Ro52, Ro60 and La48 antigens still showed minor levels (1-7%) of IgG^+ memory B cells specific for Ro52, Ro60 and La48 in their PB. In addition, 5 patients (representing all 4 FS groups) showed the common symptoms of pSS, that is dry eyes and dry mouth (Table I).

CD20-positive B cell zones and CD27/CD20 double-positive memory B cell pattern in SG of pSS patients

CD27 is a common marker present on memory B cells, plasma blasts and plasma cells. Still, both plasma blasts and plasma cells are CD20 negative, while memory B cells express CD20. As a result, the distinction of double-positive memory B cells expressing CD27 and CD20 was made possible after double-staining the SG biopsies with these surface markers. In addition, by staining the SG sections with CD20, we managed to also identify the BCZ within the gland. Taking into consideration that our study group represented FS values 0 to 3, we managed to identify a pattern for the glandular BCZ and memory B cells in relation to the FS. Patients with FS = 0 had no BCZ, and therefore served as a negative control, while those patients in the other groups (FS = 1, FS = 2 and FS = 3) showed a tendency for higher BCZ number with higher FS (Figure 1A). Hence, patients with FS = 1 and FS = 2 both had a mean number of BCZ of 5 per 10 mm^2 of SG tissue, while those with FS = 3 showed an average BCZ value of 6 (Figure 1B).

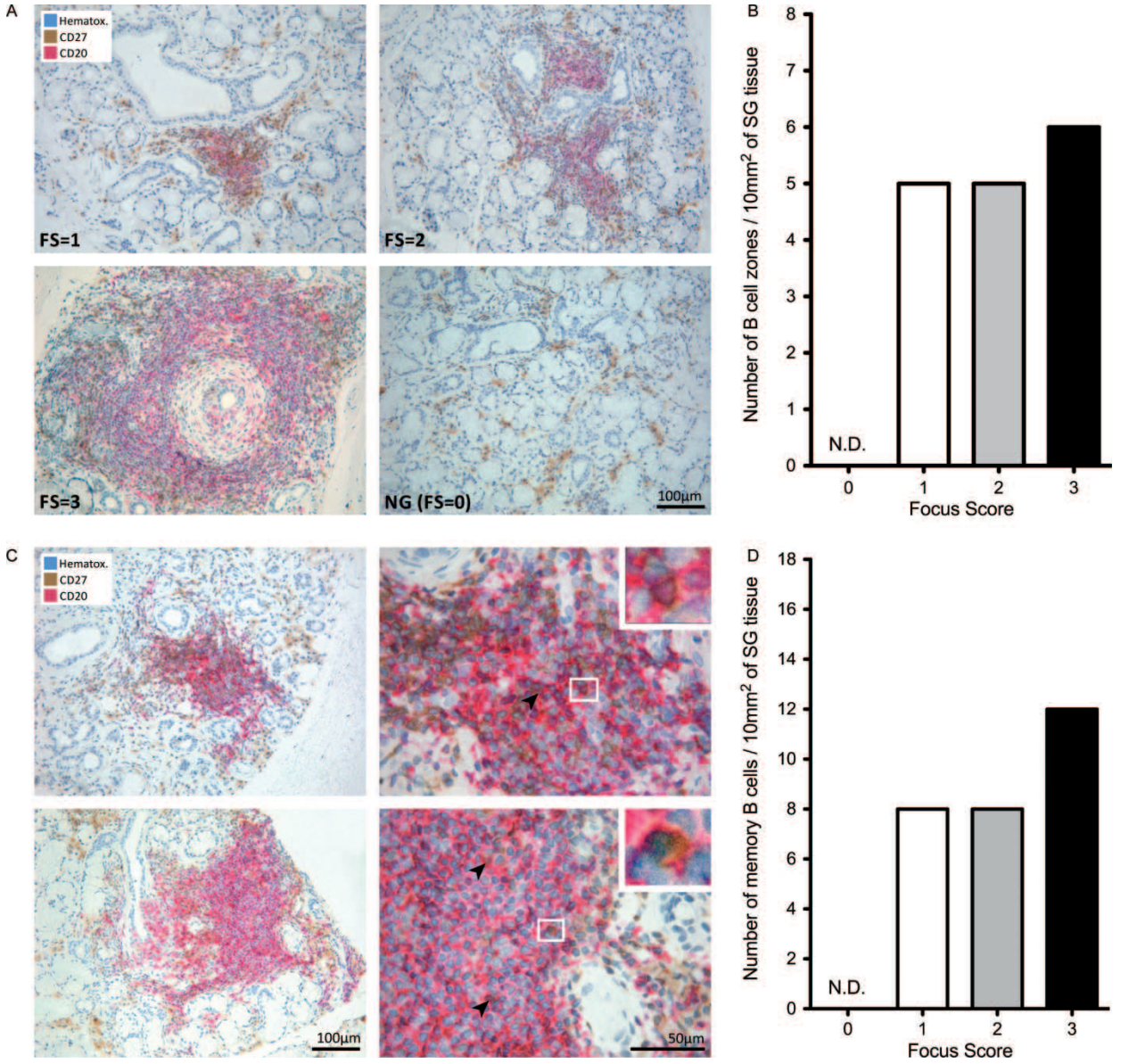

Figure 1. CD20-positive B cell zones and CD27/CD20 double-positive memory B cell pattern in SG of pSS patients. **(A)** $CD20^{+}$ B cell zones (red) in a pSS patient with FS = 1, another with FS = 2, a pSS patient with FS = 3, and a subject with normal gland (NG) histology where FS = 0. There is a general increase in glandular B cell zones (BCZ) with increasing FS, while no BCZ were observed in the patient with NG histology. **(B)** $CD20^{+}$ glandular BCZ presented as mean number of BCZ per 10 mm^2 of SG tissue for each FS group. The white bar represents FS = 1, the grey bar represents FS = 2, and the black bar represents FS = 3. N.D. = non-detectable. **(C)** Low number of double-positive memory B cells expressing both CD27 (brown) and CD20 (red). Plasma blasts and plasma cells are detected as single-positive cells expressing $CD27^{++}$ (brown). Other naïve B cells that are single-positive for CD20 and are infiltrating the BCZ are also detected (red). (**D**) CD27/CD20 double-positive memory B cells presented as mean number of memory B cells per 10 mm^2 of SG tissue for each FS group. The white bar represents FS = 1, the grey bar represents FS = 2, and the black bar represents FS = 3.

After identifying the CD27/CD20 double-positive memory B cells in the SG of the pSS patients, we noticed that the number of memory B cells observed was very low in all BCZ-positive patients. This is particularly apparent when comparing the low number of memory B cells observed to the high number of plasma blasts and plasma cells detected, that also express CD27 yet no CD20, and other naïve B cells that are CD20 positive (Figure 1C).

Nonetheless, after counting these CD20/CD27 double-positive cells we observed an increase in memory B cells with higher FS. Patients with FS = 0 and no glandular BCZ subsequently showed no memory B cells and served as a negative control once again. On the other hand, patients with FS = 1 and FS = 2 had a mean number of memory B cells of 8 per 10 mm^2 of SG tissue, while patients with FS = 3 had an average memory B cell count of 12 (Figure 1D).

Consequently, by applying Pearson correlation test we examined the co-variation between the FS and the mean number of both memory B cells and BCZ per 10 mm^2 of SG tissue. We found an association

between the FS and mean number of memory B cells ($r^2 = 0.5110$, $P = 0.0202$), and also the FS and mean number of BCZ ($r^2 = 0.4604$, $P = 0.0310$). Moreover, a correlation was also found between the total number of BCZ and the total number of memory B cells per 10 mm^2 of SG tissue ($r^2 = 0.6483$, $P = 0.0049$) as presented through linear regression (Figure 2).

Plasma cells expressing CD27 and CD138 in SG of pSS patients

CD138 is a marker for both plasma cells and long lived plasma cells, yet is absent on plasma blasts. Therefore, to distinguish the CD27^{++}/CD20^{-} plasma blasts, from the CD27^{++}/CD20^{-} plasma cells, single-staining with CD138 was carried out on serial sections from the SG of the same patients. A high number of CD138-positive plasma cells and long-lived plasma cells was observed in all SG tissue, yet to a lesser extent in the control group with FS = 0.

Interestingly, cells that were positive for both CD27^{++} in the double-staining experiment and CD138 in the single-staining experiment were usually located *outside* the BCZ, and were observed interstitially and in clusters. This indicated that these cells were either plasma cells or long-lived plasma cells. The other CD27^{++}/CD20^{-} cells that were negative for CD138 and were observed *within* the BCZ. This implies that plasma blasts tend to be situated within the BCZ, while the plasma cells and long-lived plasma cells are generally located outside the BCZ of SG tissue from pSS patients (Figure 3). To verify our observations, additional staining with CD19 was also carried out because it targets a greater population of B cells in addition to the BCZ (data not shown).

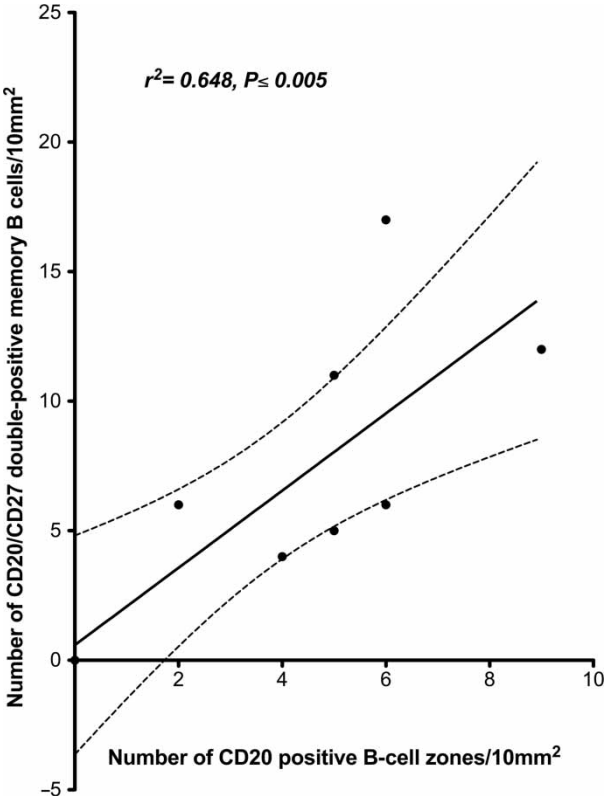

Figure 2. Correlation between the total number of CD20^{+} BCZ, and the total number of CD27/CD20 memory B cells per 10 mm^2 of SG tissue. The number of glandular BCZ per 10 mm^2 of SG tissue is plotted on the x-axis, while the number of memory B cells is presented on the y-axis. r = Pearson's correlation coefficient.

Discussion and Conclusion

In this study, we identified the CD20-positive BCZ and CD27/CD20 double-positive memory B cells by examining formalin fixed, paraffin-embedded minor SG tissue from 10 pSS patients. This allowed the study of the glandular B cell pattern and the subsequent characterisation of memory B cells in the SG of these individuals. We have previously observed a significant reduction of Ro and La specific memory B cells in the PB of these same subjects [45]. This was consistent with what had been reported earlier on memory B cell pattern in PB of pSS patients, stating that there is usually a depressed percentage of circulating memory B cells in pSS [10,11,43]. It was therefore of particular interest to explore the implications these diminished levels of memory B cells in the PB of pSS patients had on the number of memory B cells infiltrating their SG tissue.

It was previously reported that diminished levels of memory B cells in PB resulted in the accumulation of these cells in the SG of SS patients [43]. This was shown by the IF staining of parotid tissue obtained from 1 SS patient that was also suffering from lymphoma, a diagnosis that could have a profound affect on B cell pattern in this individual. For these reasons, we decided to test whether this claim on retention of memory B cells in the SG of SS patients is indeed reliable in our patient material. We applied IHC staining to SG tissue from 10 subjects diagnosed with primary SS.

In this way, one could exclude the affect of other associated rheumatic diseases on the pattern observed in the SG of these individuals. In addition, by applying double IHC staining with CD27 and CD20 on paraffin embedded tissue sections of SG we also managed to gain insight on the morphology and distribution of these cells within the gland. Together, our findings could in turn give insight into the efficiency of current therapeutic strategies that rely on depletion of CD20-positive cells in SS patients [47].

When comparing the re-evaluated FS values with the mean number of BCZ and memory B cells per 10 mm^2 of SG tissue, respectively, a similar pattern was observed. In both instances, no BCZ nor memory B cells were detected when the FS was zero. This was expected, since this particular group served as our

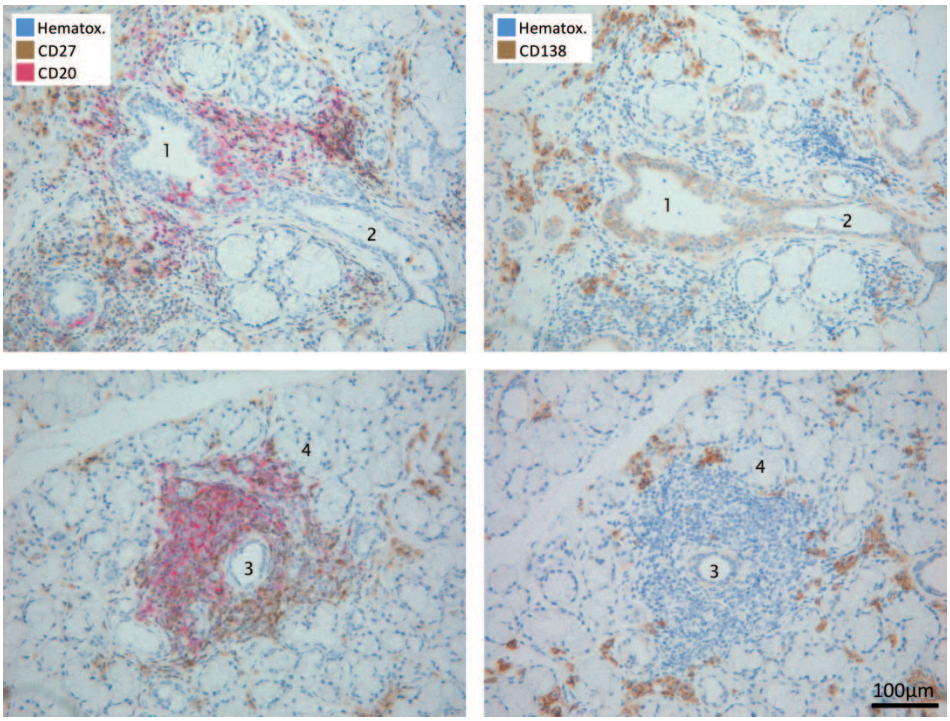

Figure 3. Plasma cells expressing CD27 and CD138 in SG tissue from two different pSS patients. A double-staining identifying $CD27^{++}$ plasma blasts (brown) and plasma cells (brown), in addition to $CD20^{+}$ glandular BCZ (red). This is followed by a single-staining of serial sections to identify $CD138^{+}$ plasma cells (brown) and long lived plasma cells (brown). The same structures in the left and right sections are marked with numbers 1, 2, 3 and 4. Cells observed as brown in both single- and double-staining experiments are subsequently $CD27^{++}/CD138^{+}$ plasma cells, while those observed in the double-staining but not in the single-staining are $CD27^{++}/CD138^{-}$ plasma blasts. CD138 is also expressed on long-lived plasma cells as seen in the single-staining experiment.

non-pSS tissue control in the study, while those patients in the other groups (FS = 1, FS = 2 and FS = 3) showed an increase in BCZ as the FS increased (Figure 1A).

Furthermore, there was little difference in the average number of BCZ and memory B cells when the FS was 1 and 2. On the other hand, both memory B cells and BCZ means increased when the FS was 3 (Figure 1B and 1D). This suggests that increased mononuclear infiltration could subsequently lead to a higher number of BCZ and memory B cells in SG tissue. However, individual variation was observed, since other factors may also influence this particular pattern such as chemokines and cytokines within the inflamed tissue [48]. In addition to this, the time point at which the SG biopsies were taken in relation to disease progression for each subject might also have an affect on the patterns studied. Hence, based on the limited number of patients included in this study only a trend could be indicated.

Contrary to what had been reported previously [43], we found the total number of memory B cells infiltrating the SG to be very low in all BCZ-positive patients tested. This becomes especially evident upon comparing the low number of memory B cells observed to the high number of CD27-positive plasma blasts and plasma cells detected, in addition to other naïve B cells that are CD20-positive (Figure 1C). Hence, lower levels of Ro and La specific memory B cells in PB of these 10 pSS patients resulted nonetheless in a low number of memory B cells in their SG tissue. Also, our IHC analysis showed that the memory B cells were situated within the CD20-positive BCZ identified (Figure 1C). This explains the correlation we observed between the total number of BCZ and the total number of memory B cells per 10 mm^2 of SG tissue (Figure 2).

Furthermore, the single-staining with CD138 that was carried out on serial sections from the SG of the same patients helped us to distinguish the $CD27^{++}/CD20^{-}$ plasma blasts, from the $CD27^{++}/CD20^{-}$ plasma cells. High numbers of CD138-positive plasma cells and long-lived plasma cells were observed in all analysed SG tissue, yet to a lesser extent in the control group with FS = 0. Interestingly, plasma cells and long-lived plasma cells that were positive for both CD27 in the double-staining experiment and CD138 in the single-staining experiment were usually located *outside* the BCZ in the focal infiltrates, and were also observed in clusters within interstitial areas located close to ductal and acinar epithelia. This is in correspondence with what has been reported earlier [18]. On the other hand, $CD27^{++}/CD20^{-}$ plasma blasts that were negative

with the CD138 staining were in most cases observed *within* the BCZ (Figure 3). Still, plasma blasts, plasma cells and long-lived plasma cells were present at a much higher frequency than CD27/CD20 double-positive memory B cells in the SG tissues of all subjects.

At present, therapeutic interventions in SS depend on the targeting and elimination of CD20-positive cells by the administration of the anti-CD20 antibody Rituximab®. Still, the effects of Rituximab are usually short-term and patients may experience relapse, which results in elevated levels of autoantibodies in serum and glandular infiltrations nonetheless. Indeed, hypergammaglobulinaemia is a prominent factor in the pathogenesis of SS [9].

This was also evident in our study population and indicated by the detection of ANA in 90% of our subjects, in addition to anti-Ro and anti-La autoantibodies in patients with high FS (Table I). Given that our results have shown both plasma cells and long-lived plasma cells to be the most abundant sub-type of glandular B cells, and to be CD20-negative, they would in turn not be affected by treatment with the anti-CD20 antibody Rituximab. We suggest considering another monoclonal antibody for therapy that would target a broader population of B cells, such as anti-CD19.

In conclusion, we have shown that pSS patients with decreased levels of circulating memory B cells also display a low number of memory B cells in their SG tissue. Nonetheless, elevated numbers of plasma blasts, plasma cells, and long-lived plasma cells were also detected in these same individuals. Together our findings suggest that these lower numbers of memory B cells might be the result of the activation of these cells into CD20-negative antibody secreting plasma cells and long-lived plasma cells at the site of inflammation, which are also resistant to treatment. Further studies need to be conducted to examine whether a similar B cell pattern caused by anti-Ro and anti-La specific B cells can also be identified in the SG of pSS patients.

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