

Activation-Induced Cytidine Deaminase Expression in Follicular Dendritic Cell Networks and Interfollicular Large B Cells Supports Functionality of Ectopic Lymphoid Neogenesis in Autoimmune Sialoadenitis and MALT Lymphoma in Sjögren's Syndrome¹

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Demonstration of ectopic germinal center-like structures (GC-LSs) in chronically inflamed tissues in patients with autoimmune disorders is a relatively common finding. However, to what extent ectopic lymphoid structures behave as true GC and are able to support class switch recombination (CSR) and somatic hypermutation (SHM) of the Ig genes is still debated. In addition, no information is available on whether CSR and SHM can take place in the absence of GCs at extrafollicular sites in an ectopic lymphoid tissue. In this study, we show that in salivary glands (SGs) of Sjögren's syndrome (SS) activation-induced cytidine deaminase (AID), the enzyme responsible for CSR and SHM is invariably expressed within follicular dendritic cell (FDC) networks but is not detectable in SGs in the absence of ectopic GC-LSs, suggesting that FDC networks play an essential role in sustaining the Ag-driven B cell proliferation within SS-SGs. We also show that the recently described population of interfollicular large B cells selectively expresses AID outside ectopic GC in the T cell-rich areas of periductal aggregates. Finally, we report that AID retains its exclusive association with numerous, residual GCs in parotid SS-MALT lymphomas, whereas neoplastic marginal zone-like B cells are consistently AID negative. These results strongly support the notion that ectopic lymphoid structures in SS-SGs express the molecular machinery to support local autoantibody production and B cell expansion and may play a crucial role toward lymphomagenesis. *The Journal of Immunology*, 2007, 179: 4929–4938.

One of the hallmarks of autoimmune disorders in general, and Sjögren's syndrome (SS)⁴ in particular, is the presence of an Ag-driven activation and proliferation of B lymphocytes (1, 2). In SS, this process leads to the production of

SS-associated autoantibodies such as rheumatoid factor (RF), anti-Ro/SSA and anti-La/SSB that can be detected in the serum of most SS patients (3).

Ag-driven BCR selection via affinity maturation is believed to take place primarily in germinal centers (GCs) in secondary lymphoid organs where the processes of class switch recombination (CSR) and somatic hypermutation (SHM) of the Ig genes takes place in GC B cells (4). However, CSR and low-level SHM have also been shown to occur at extrafollicular sites in rodent secondary lymphoid organs (5). Recently, CSR and SHM have been shown to be critically dependent on the expression of activation-induced cytidine deaminase (AID) (6), an enzyme originally described to be specifically expressed in GC B cells (7). Accordingly, it has been also clearly demonstrated that AID protein expression in secondary lymphoid organs is restricted to GC B cells undergoing CSR and SHM and also to a newly identified B cell subpopulation defined as interfollicular (IF) large B cells (8–10).

We and others recently demonstrated that in minor (labial) salivary glands (mSG) of SS patients (SS-mSG) ectopic GC-like structures (GC-LS) can be detected in around 20–40% of the patients (11–13). This definition is based on the acquisition, by periductal inflammatory foci, of features of secondary lymphoid

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⁴ Abbreviations used in this paper: SS, Sjögren's syndrome; SG, salivary gland; GC-LS, germinal center-like structures; CSR, class switch recombination; SHM, somatic

hypermutation; mSG, minor (labial) salivary glands; FDC, follicular dendritic cells; RF, rheumatoid factor; IF, interfollicular; MZB, marginal zone; FoB, follicular B cells; NSCS, nonspecific chronic sialoadenitis; LESA, lympho-epithelial sialoadenitis; IHC, immunohistochemistry; AID, activation-induced cytidine deaminase; LN, lymph node.

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Table I. Demographic and laboratory characteristics of the SS patients [primary SS (pSS), secondary SS (sSS)] enrolled in this study

	SS mSG	SS Parotids	SS MALT-L
Number of patients	22	5	18
pSS/sSS	19 pSS/3 sSS (RA)	5 pSS	14 pSS/4 sSS (3 RA, 1 SLE)
Gender	20 F/2 M	4 F/1 M	16 F/2 M
Age (mean range, years)	53.3 (21–72)	52 (48–56)	54.3 (30–74)
Disease duration (mean range, months)	77.6 (24–204)	64 (48–72)	88.3 (36–204)
RF (%)	68.2%	80%	90.9% ^a
Anti/SSA-SSB (%)	50%	60%	81.8% ^a
ANA (%)	72.3%	80%	90.9% ^a

^a Data available from 11 patients.

organs such as T/B cell compartmentalization, follicular dendritic cell (FDC) network formation and peripheral node addressin expression (11, 12), a phenomenon known as ectopic lymphoid neogenesis (14, 15). Although indirect evidence such as the association of GC-LS with the presence of B cells reacting with Ro/La proteins in situ (13) and the demonstration of an Ag-driven B cell response within ectopic GC in SS-mSGs (2) have been provided, the functionality of these ectopic GC-LS in SS in actively supporting CSR and SHM is still debated (16).

Chronic Ag-driven polyclonal B cell activation in SS has been suggested to favor selection and expansion of autoreactive B cell clones via CSR and SHM (2). The inherent genetic instability associated with DNA hypermutation can lead, in up to 5% of SS patients, to the escape of malignant B cell clones leading to development of a B cell lymphoma which typically is a low-grade marginal zone (MZ) lymphoma of the MALT (MALT-L) (17). Malignant MZB cells in SS are clonally related to the nonlymphomatous autoreactive B cells (18) suggesting that the local reactive Ag-driven immune response is responsible for stimulation of lymphomagenesis (19). In addition, high levels of SHM and even intracanal variation (20, 21) have been reported in malignant MZB cells in parotid MALT-Ls suggesting that Ag-driven selection of malignant B cell clones is still active in established low-grade SS-MALT-Ls.

However, whether malignant MZB cells are capable of expressing AID in SS-MALT-Ls is unclear. In this report, we provide evidence that AID is invariably expressed ectopically in SS-mSG and parotids within periductal foci characterized by T/B cell segregation and FDC networks. In addition, we show that outside GC-LSs AID expression identifies a population of B cells with the morphology of the recently described IF large B cells. Finally, we demonstrate that in SS-MALT-Ls AID expression is consistently absent in malignant MZB cells while it is abundantly and selectively expressed by GC B cells within the residual reactive component of the MALT-L characterized by CD21⁺ FDC networks.

Materials and Methods

Patients and samples

All patients fulfilled the revised criteria of the American-European Consensus Group (22) for either primary (pSS) or secondary Sjogren's syndrome (sSS). Demographic and main laboratory characteristics are described in Table I. Biopsies from mSG of 10 patients with nonspecific chronic sialoadenitis (NSCS) (8 females and 2 males, mean age 56.5; range 33 to 71; mean disease duration 72-mo range (24–120 mo)), were chosen as control group. Normal human lymph nodes (LNs) obtained from patients undergoing vascular surgery were used for comparison. Procedures were performed after informed consent approved by the hospital Ethics Committee (REC 05/Q0702/1).

Formaldehyde-fixed, paraffin-embedded tissue samples were obtained from mSG biopsies of 22 SS patients with focus score ≥ 1 (23). In addition, parotid samples of 5 SS patients) with lympho-epithelial sialoadenitis (LESA) and 18 SS patients with low-grade MALT lymphoma (SS-

MALT-L) were selected from the biopsy bank of the Department of Oral Pathology at Guy's Hospital. Histologically, MALT-L were diagnosed by the presence of sheets or halos of monocytoid B cells, most often infiltrating epimyoeplithelial islands. The existence of clonal populations was confirmed by VDJ PCR for the H chain Ig genes. Of the 18 patients with SS-MALT-L, previous (average 3.3 years, range 1–9) mSG biopsies collected between 1989 and 2002 were available from eight patients.

Fresh parotid samples were obtained from 2 patients with SS and LESAs and 3 patients with SS and MALT-L undergoing diagnostic procedures (2 parotidectomy, 3 parotid tail biopsies) whereas control glands were obtained from 2 patients with squamous cell carcinoma and 1 normal parotid. Freshly collected parotid glands were part embedded in paraffin for immunohistochemistry (IHC) and part dissected in multiple pieces and stored in a 10:1 v/v of RNALater (Ambion) at -80°C for TaqMan RT-PCR analysis.

Immunohistochemistry

A list of primary and secondary Abs used is reported in Table II.

Grading analysis, histological characterization of lymphoid proliferation and AID expression in mSG of SS patients

The cellular infiltrate and degree of lymphoid organization were assessed by immunohistochemical staining of sequential sections of mSGs with Abs to CD3, CD20, CD21, and peripheral node addressin (Meca-79) as previously reported (11, 12). Briefly after de-waxing and re-hydration formalin-fixed, paraffin-embedded 3- μm thick sections underwent appropriate Ag retrieval with Target retrieval solution or proteinase K (DakoCytomation). Double staining for CD3 and CD20 was used to analyze T/B cell segregation using the Dako EnVision Doublestain system (DakoCytomation) as previously reported (11, 12).

Based on the results of the double staining, a scoring system based on the size and degree of organization of infiltrates was used (11, 12). Cellular aggregates with periductal lymphocytes numbering between 10 and 50 were defined as grade 1 aggregates (G1), grade 2 aggregates (G2) comprised >50 periductal lymphocytes whereas grade 3 aggregates (G3) were characterized by >50 periductal lymphocytes plus the presence of GC-LSs. In addition, on the basis of the CD3/CD20 double staining, lymphocytic G2 foci were classified as 1) nonsegregated (NS-G2), when no clear compartmentalization of T and B cells in discrete areas was recognized and 2) segregated (S-G2), when the foci displayed separated T and B cell-rich areas. The presence of GC-LSs was determined by the presence of T and B lymphocytes and CD21⁺FDC networks on sequentially stained sections (2, 11, 12). To minimize any bias due to the cutting level, some follicles were cut and stained every 50 μm . Staining for AID was performed on sequential sections of the above to correlate the degree of follicular organization with AID expression. After Ag unmasking using Target retrieval solution (pH 6; DakoCytomation) and incubation with protein block solution (DakoCytomation) endogenous biotin was blocked with the avidin-biotin blocking system (DakoCytomation). Primary Ab EK2-5G9 was incubated at an appropriate dilution for 1 h at room temperature and biotinylated rabbit anti-rat Ig was used as a secondary Ab. Following a 1-h incubation and three washes, HRP or AP-streptavidin-biotin complex (DakoCytomation) was added to the section and incubated for 30 min. After further washes color reaction was developed with diaminobenzidine (HRP, DAB+; DakoCytomation) or Vector red (for AP; Vector Laboratories). Double staining for AID and the cellular markers CD20 and CD3 was performed to confirm the cellular source of AID⁺ cells. Aggregates were considered positive for AID when at least three positive cells were stained.

Table II. List of primary and secondary antibodies used for immunohistochemistry and primers and probes for Taqman real-time PCR

Clone/Name	Specificity	Host	Source
Primary Abs			
L-26	Human CD20	Mouse	DakoCytomation
A2452	Human CD3	Rabbit	DakoCytomation
	Human CD21		
1F8	Human bcl-2	Mouse	DakoCytomation
124	Human IgD	Mouse	DakoCytomation
IgD	Human bcl-6	Rabbit	DakoCytomation
PG-B6p	Human CD31	Mouse	DakoCytomation
1A10	Human/mouse peripheral node addressin	Mouse	Novocastra
MECA-79	Human AID	Rat	BD Pharmingen
EK2-5G9		Rat	Ref. 9
Secondary Abs			
Rabbit anti-rat biotin	Rat Ig	Rabbit (biotinylated)	DakoCytomation
Goat anti-mouse biotin	Mouse Ig	Goat (biotinylated)	DakoCytomation
EnVision AP/HRP	Mouse/rabbit Ig		DakoCytomation
Gene product	mRNA accession number		Source
Human AID	NM_020661		Applied Biosystems
Human CD21L	NM_001006658		Applied Biosystems
Human β -actin	NM_001101		Applied Biosystems

Quantitative Taqman real-time PCR

Total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen), with on column DNase I digestion to avoid genomic DNA contamination. First-strand cDNA was synthesized from 500 ng of total RNA with a Thermo-script RT-PCR System for first-strand cDNA synthesis (Invitrogen Life Technologies). For quantitative Taqman real-time evaluation of AID, CD21 long isoform (CD21L, specific for FDC (24)) primers and probes were obtained from Applied Biosystems (Table II). Samples were run in triplicate at 20 ng of cDNA/well, detected using the ABI PRISM 7900HT instrument and results analyzed using the ABI PRISM 7900HT sequence detection system version 2.1. Relative quantification was assessed using the comparative C_T (threshold cycle) method with cDNA from normal human lymph nodes used for calibration.

Histological characterization of the lymphoid proliferation in SS patients with MALT-Ls and relationship with AID expression

To characterize B cell subpopulations within SS parotids with LESA and SS-MALT-L, in addition to double staining for CD3/CD20 (T/B cell segregation), parotid sections were double stained for CD20/IgD and CD21/bcl-2 and single staining for bcl-6 to identify, together with morphological analysis, follicular, GC and marginal zone-like B cells. AID expression was then evaluated on sequential sections.

Statistical analysis

Differences in quantitative variables were analyzed by the Mann-Whitney U test. χ^2 test with Yates' correction when required or Fisher's exact test

when appropriate were used to evaluate associations of qualitative variables in the different groups. Spearman's rank correlation was performed to correlate expression levels of AID and CD21L mRNA in SS parotids. All the statistical analyses were performed using GraphPad Prism version 3.03 for Windows. A value of $p < 0.05$ was considered statistically significant.

Results

Histomorphological grading, T-B cell compartmentalization and formation of FDC networks in SS-mSGs and SS-parotids

In SS-mSGs the total number of aggregates (mean \pm SEM) at three consecutive cutting levels (50 μ m a part was 254 ± 5 ; the majority were G2 aggregates (60.2%; 153.3 ± 5.5), a smaller percentage were G1 (33.5%; 85.5 ± 3.5) and fewer were G3 foci (6.3%; 16.5 ± 2.4). These graded aggregates were further analyzed by double staining with anti-CD3/CD20 and anti-CD21/bcl-2 Abs for the presence of T/B cell segregation and FDC networks. As we have previously reported (11), G1 aggregates were populated by a predominance of CD3⁺ lymphocytes with fewer B cells (Fig. 1A) whereas G2 and G3 foci were characterized by increased number of B cells. In addition, although most G2 aggregates demonstrated no obvious compartmentalization in B and T cell areas (Fig. 1B), 32.4% of the G2 showed a clear segregation of B and T cells in discrete areas (Fig. 1C). All G3 foci

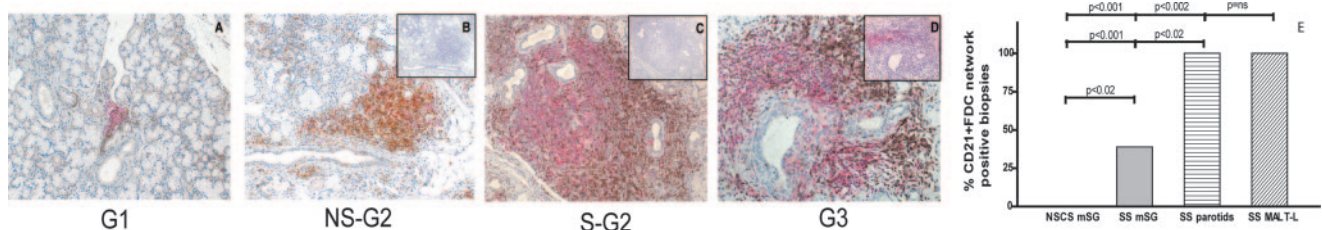


FIGURE 1. Characterization of the cellular infiltrates and degree of lymphoid organization in mSG from patients with SS. Sequential paraffin-embedded sections of SS-mSG patients were double stained for T (CD3, brown) and B lymphocytes (CD20, purple) (A–D) and single stained for CD21 (purple) to detect CD21⁺ FDC networks (B–D insets). Periductal inflammatory foci were classified into four histologic groups according to the size and the degree of lymphoid organization based on the evidence of compartmentalization of T and B cells in discrete areas and presence of networks of CD21⁺ FDCs to identify GC-LSs. Representative examples of grade 1 (G1; <50 periductal lymphocytes) (A), nonsegregated grade 2 (NS-G2; >50 periductal lymphocytes with no evidence of T/B cell segregation) (B), segregated grade 2 (S-G2; >50 periductal lymphocytes with evidence of T/B cell segregation) (C), and grade 3 (G3; >50 periductal lymphocytes, with presence of CD21⁺ FDC network as marker of GC-LSs) (D) are shown (original magnification, $\times 100$). E, Prevalence of salivary gland biopsies with CD21⁺ FDC networks characterizing G3 aggregates with GC-LS in SS-mSG, SS parotids and SS-MALT-Ls as compared with NSCS.

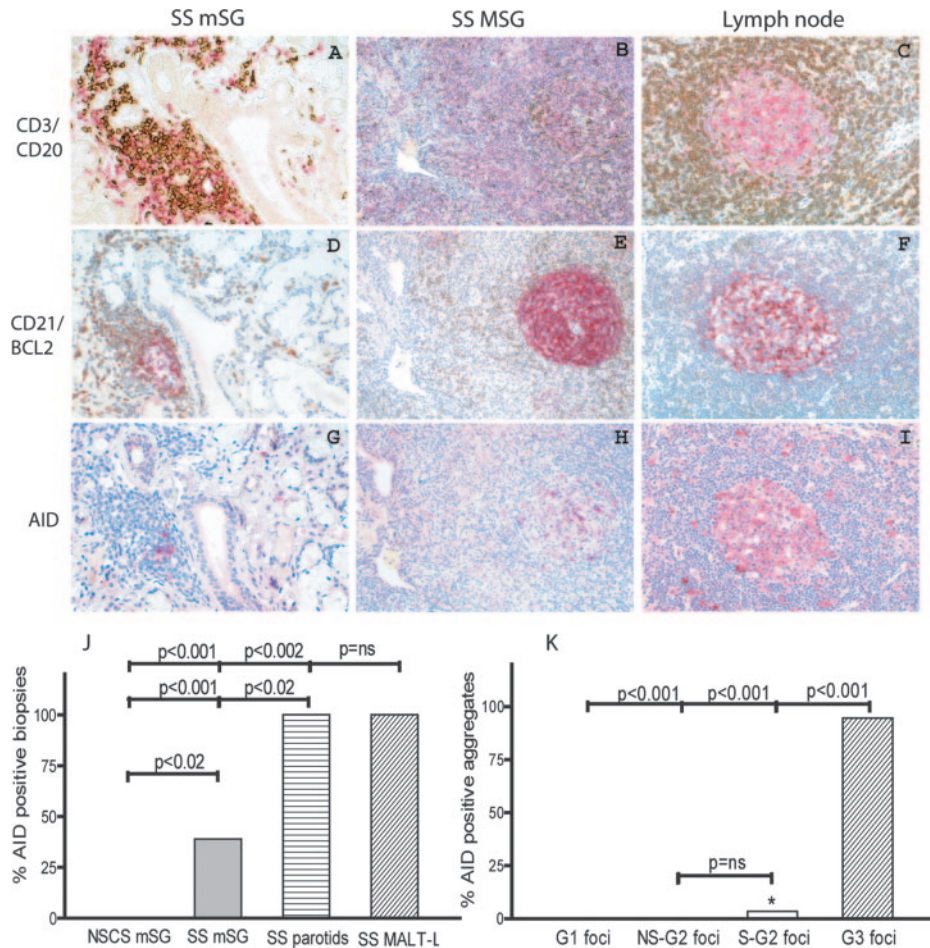


FIGURE 2. AID is invariably expressed within GC-LSs in mSG and parotids in patients with SS. Sequential paraffin-embedded sections from SS patients were double stained for CD3 (brown) and CD20 (purple) (A–C), for bcl-2 (brown) and CD21 (purple) (D–F) and single stained for AID (purple) (G–I). Representative examples of SS mSG (A, D, G) and parotids (B, E, H) are shown together with a mature lymphoid follicle in a lymph node (C, F, I) for comparison. Presence of CD21⁺FDC network (D) is invariably associated with AID expression (G) that can be observed even in relatively small periductal aggregates and in the absence of clear T/B cell segregation (A) in SS-mSG. AID expression is more prominent (H) in larger GC-LS (E) in SS-parotids and similar to the expression pattern observed in secondary lymphoid organs (I) (original magnification, $\times 200$ in A, D, G and $\times 100$ in B, E, H and C, F, I). J, Prevalence of AID expression in mSG of patients with NCS, SS-mSG, SS-parotids, and SS-MALT-L parotid biopsies from SS patients. K, Prevalence of AID expression in periductal inflammatory foci characterized by increasing degrees of lymphoid organization (G1 to G3) in mSGs of SS suggests that the presence of CD21⁺FDC networks sustains AID expression. *, scattered AID-positive B cells with the appearance of interfollicular large B cells observed in inflammatory foci in close association with numerous GC-LSs in a labial SG biopsy from a single SS patient.

displayed features of mature secondary lymphoid organ follicles with a central duct surrounded by B cells and CD21⁺ FDC networks forming GC-LS bordered by T lymphocytes (Fig. 1D).

In SS-mSG the presence of G1 and G2 aggregates was found in 100% of patients whereas G3 foci were found in 8 of 22 patients (36.3%, Fig. 1E). Conversely, in the NCS cases evaluated, G1 aggregates were detected in 70% of the patients (SS vs NCS, $p = ns$), G2 aggregates were detected in 10% ($p < 0.001$) whereas, as previously reported (11, 12), no G3 aggregates were detected in NCS patients ($p < 0.04$, Fig. 1E).

In comparison with SS-mSGs, SS-parotids and SS-MALTs displayed a higher prevalence of G3 aggregates (100% of the sample analyzed, $p > 0.02$ and $p > 0.002$ for SS-parotids and SS-MALT-Ls vs mSG, respectively, Fig. 1E) with larger and more numerous CD21⁺GC-LSs.

AID expression is invariably associated with the presence of FDC networks in ectopic germinal centers in SS mSGs and parotids

Sequential section analysis in SS-mSGs identified the precise and exclusive colocalization of AID expressing cells within GC-LSs

characterized by CD21⁺FDC networks even in the absence of a morphologically defined GC (Fig. 2, A, D, and G). AID was mainly localized in the cytoplasm with a minority of B cells also expressing nuclear AID, in line with previous reports in secondary lymphoid organs (9, 10). The number of AID⁺ cells within GC-LSs in SS-mSGs was generally lower as compared with SS-parotids, where larger GCs were observed (Fig. 2, B, E, H, and I). Importantly, within GC-LSs, B cells were consistently negative for bcl-2 (Fig. 2, D and E) and positive for bcl-6 (not shown) confirming that AID⁺ cells belong to the GC B cell population as seen in lymph nodes (Fig. 2F). The distribution of AID⁺ cells within GC-LSs in SS-mSGs and SS-parotids also closely resembled that seen in GCs of secondary lymphoid organs (Fig. 2, C, F, and I).

We then quantified the prevalence of AID⁺ aggregates in the salivary glands of the different populations studied. Positive staining for AID was observed in 8 of 22 SS-mSG biopsies but not in NCS ($p < 0.0001$, Fig. 2J) and exclusively in samples where CD21⁺ FDC networks were present. In SS-mSGs, AID was not observed either in G1 or G2 foci, either segregated or not, suggesting that the presence of FDC networks is required for AID expression in B cells in ectopic lymphoid neogenesis. Conversely,

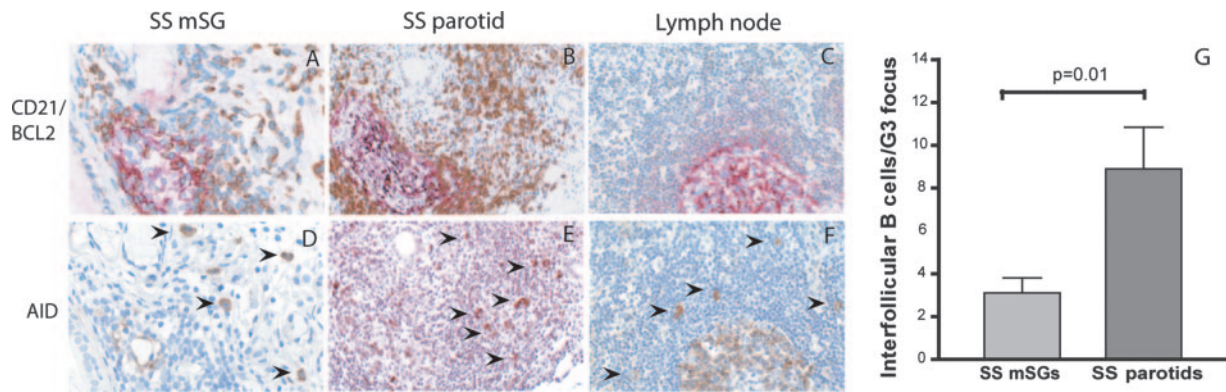


FIGURE 3. AID expression in SS-mSG and SS-parotids also identifies cells resembling the recently described population of IF large B cells. Sequential paraffin-embedded sections from SS patients were double stained for bcl-2 (brown) and CD21 (purple) (A–C) and single stained for AID (brown) (D–F). Representative examples of SS mSG (A–D) and parotids (B–E) are shown with a tertiary lymphoid follicle in a lymph node (C–F) for comparison. AID⁺ cells with large cytoplasm and sometimes dendritic morphology (arrowheads, D–F) were frequently and exclusively observed in SS salivary glands characterized by the presence of G3 foci with GC-LSs, often in close proximity with CD21⁺FDC networks (D, E). The morphology and pattern of distribution are highly reminiscent of the population of IF large B cells recently described in secondary lymphoid organs as shown in lymph node (F). The number of IF large B cells within G3 foci and surrounding FDC networks was significantly higher in SS parotids as compared with SS mSG (G) (original magnification, $\times 200$ in A–D and $\times 100$ in B–E and C–F).

all but one G3 foci ($p < 0.0001$ vs G1 and G2) stained positively for AID, suggesting that the presence of FDC networks is sufficient to support AID expression (Fig. 2K).

AID expression also identifies IF large B cells surrounding GCs in mSGs and parotids of SS

In addition to GC B cells within FDC networks, a second pattern of AID expression was observed in mSGs and parotids of SS patients. As shown in Fig. 3, numerous large B cells often with dendritic morphology strongly expressed AID, mainly in the cytoplasm and to a lesser extent in the nucleus, and were localized in close association with CD21⁺AID⁺GCs in SS-mSGs (Fig. 3, A and D) and SS-parotids (Fig. 3, B and E). Importantly, this population was exclusively observed in SGs where G3 foci with CD21⁺ FDC networks were present. Interestingly, in SS-mSGs characterized by numerous G3 foci, IF B cells were also found in adjacent smaller aggregates lacking GC-LSs, possibly related to the ability of these cells to migrate within the glands. This pattern of expression is highly similar to that observed in secondary lymphoid organs (Fig. 3, C and F) where AID⁺ IF large B cells are mainly localized within the T cell area surrounding follicular structures, as previously reported (9, 10). Double staining of IF B cells with CD20 confirmed that they belonged to the B cell population (not shown). The number of GC-associated IF large B cells was significantly higher in SS-parotids as compared with SS-mSGs (Fig. 3G), consistent with the larger size of GCs in SS parotids.

Prevalence of AID⁺ FDC networks in SS-mSG and circulating autoantibodies in patients with SS

A clear tendency, although not statistically significant, toward increased prevalence of anti-Ro and anti-La Abs was observed comparing SS patients who displayed AID⁺ inflammatory infiltrates characterized by GCs within mSGs to SS patients without AID⁺GC in mSGs (75% vs 35.8% $p = 0.07$), RF (87.5% vs 57%), and ANA (87.5% vs 64.3%). These data strengthen the functional importance of AID expression in an ectopic setting.

AID mRNA transcripts levels are markedly over-expressed in SS-parotids with LESA and MALT-L and closely correlate with CD21L mRNA

To quantitatively analyze the expression levels of AID in SS-parotids and SS-MALT-Ls compared with secondary lymphoid or-

gans and control parotids, we performed quantitative Taqman real-time PCR. AID mRNA transcripts were strongly expressed in all fragments from both SS-parotids and SS-MALT-Ls (Fig. 4A). Interestingly, AID levels in all SS parotids were similar or higher in comparison to normal lymph nodes, suggesting that the SS parotid ectopic lymphoid infiltrate is characterized by the induction of a molecular program with the capacity to support a locally active humoral immune response. Expression of AID was not detected in any fragments obtained from control parotids (Fig. 4, A and B). Importantly, in the parotids examined, AID transcripts closely correlated (Spearman $r = 0.88$, $p > 0.0001$, Fig. 4C) with the expression levels of CD21L, an isoform of CD21 that is specifically expressed in FDCs (24). This evidence further substantiates that AID expression in SS parotids is dependent on CD21⁺FDCs.

AID protein is exclusively expressed within residual GCs and IF large B cells in SS-MALT-L

We then characterized the different B cell subpopulations responsible for aberrant AID expression in SS parotids with LESA and MALT-L. Two distinct areas of cellular organization with peculiar histological features could be identified within SS-MALT-Ls (25). Numerous follicular structures, characterized by T/B cell segregation (Fig. 5, A and B) and FDC networks (Fig. 5, C and D), were detected within 100% of the samples analyzed (see Fig. 1E). These areas in SS-MALT-Ls were defined as the *reactive* component of the lymphoid proliferation and were mainly inhabited by GC B cells within FDC networks surrounded by CD20⁺IgD⁺/bcl-2⁺ follicular B (FoB) cells (Fig. 5, G and H). Conversely, MZ-like B cells expressed a different phenotype (CD20⁺IgD⁻/bcl-2⁺) (17, 25) with peculiar histological features (monocytoid-like morphology with pale nuclei, evident nucleoli and frequent mitotic figures) and were distributed in dense sheets within the parenchyma and often infiltrated and subverted the ducts. The areas inhabited by this cell population were defined as the *malignant* component of the MALT-L, based on the morphological features, surface Ags and L chain restriction analysis.

Notably, in SS-MALT-L parotids AID was exclusively expressed within the reactive but not the malignant areas (Fig. 5, E and F). Notably, as shown in Fig. 5, AID was only detected in areas characterized by clear T/B cell segregation with CD21⁺ FDC networks forming GCs (Fig. 5, C and D), surrounded by CD20⁺IgD⁺/bcl-2⁺ FoB cells (Fig. 5, G and H). AID⁺ B cells resembling

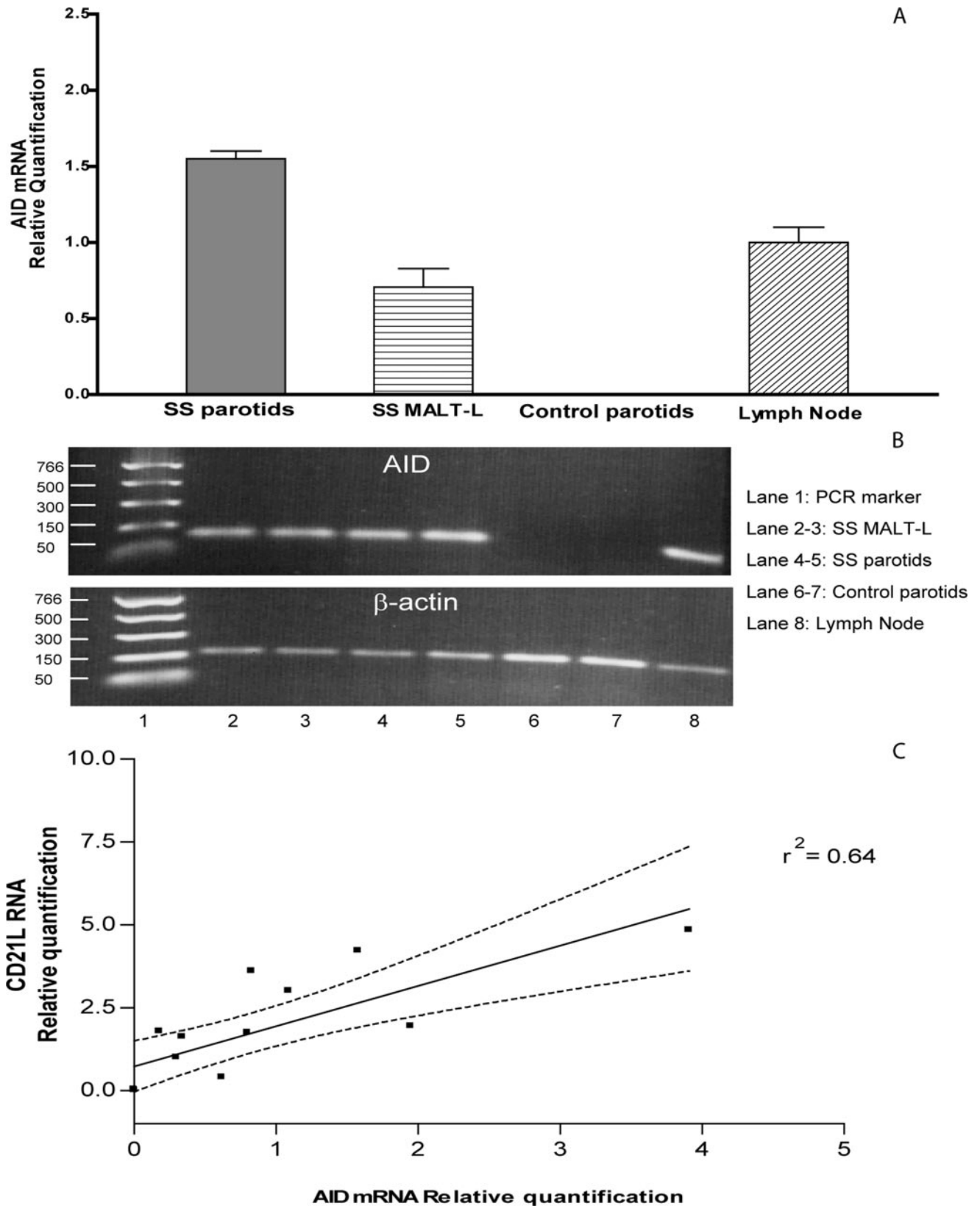


FIGURE 4. AID mRNA in SS parotid glands with LESA and MALT-L closely correlated with CD21L and is expressed at similar levels to secondary lymphoid organs. **A**, Quantitative Taqman real-time PCR was used to measure parotid AID mRNA levels in reactive parotids and MALT-L samples from patients with SS and in control parotids. After normalization for the endogenous control (human β -actin) results were expressed as relative quantification to AID mRNA expression in normal lymph nodes which was set as one. **B**, Representative examples of the PCR products (10 μ l for AID and 1 μ l for β -actin) were run in 1.8% TBE-agarose gel to ensure the presence of a single specific amplification product and confirm specificity of TaqMan real-time PCR. Amplicons of the expected length (99 bp for AID, *top gel* and 171 bp for β -actin, *bottom gel*) were observed in all SS parotids with no AID mRNA expression detectable in controls. **C**, Linear regression showing strong positive correlation between expression levels of AID and CD21L mRNA in SS parotid samples with LESA and MALT-L. See text for statistical analysis.

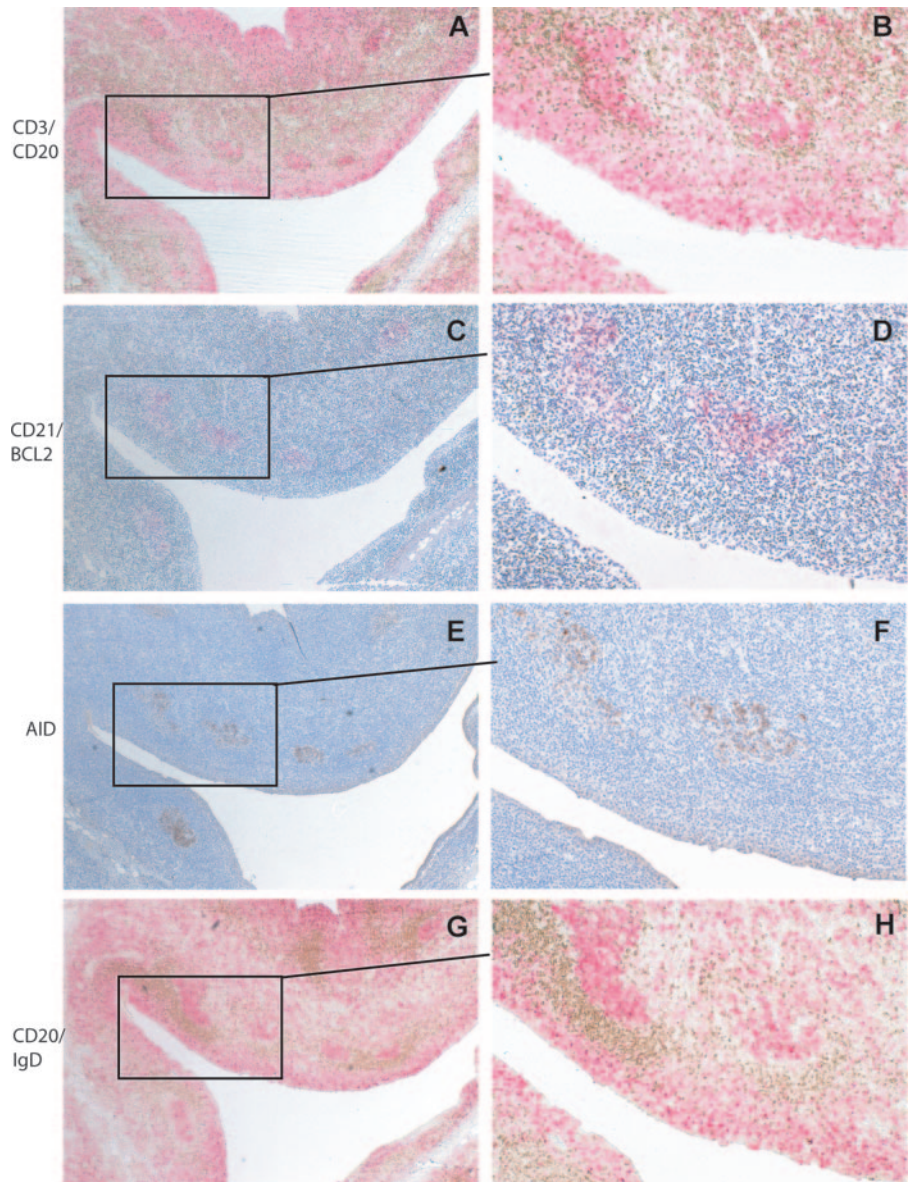


FIGURE 5. AID expression in parotid MALT-L in patients with SS retains its association with residual GC-LSs but is not detectable in MZ-like B cells infiltrating the ducts. Representative example of a low-grade MALT-L arising in a parotid gland of a patient with SS. Sequential paraffin-embedded sections were double stained for CD3 (brown) and CD20 (purple) (A, B), for bcl-2 (brown) and CD21 (purple) (C, D), single stained for AID (brown) (E, F) and double stained for IgD (brown) and CD20 (purple) (G, H) to define, together with the morphological analysis, the residual reactive and malignant components of the MALT-L. AID was exclusively expressed in residual GCs characterized by T/B segregation (A, B) and CD21⁺ FDC networks (C, D) and surrounded by CD20⁺/IgD⁺/bcl-2⁺ follicular B cells (G, H). Conversely, AID was not detectable among the population of CD20⁺/IgD⁻/bcl-2⁺ MZ-like B cells infiltrating residual ductal structures (G, H) (original magnification, $\times 40$ in A, C, E, G and $\times 200$ in B, D, F, H).

IF large B cells were also detected in some but not all SS MALT-L, most often in association with the T cell area. GCs in SS-MALT-Ls were most often closely associated with ductal structures and CD21⁺ FDC networks sometimes surrounded a central duct. Conversely, the dense sheet of malignant MZ-like CD20⁺/IgD⁻/bcl-2⁺ B cells that completely infiltrated residual duct were consistently negative for AID expression in all the samples analyzed (Fig. 5, E and F).

Prevalence of ectopic germinal centers in SS mSG biopsies is increased in patients who subsequently develop parotid MALT-L

Analysis of serial biopsies within parotid glands has been previously used to demonstrate clonal relationship between the prelymphomatous stage and subsequent malignant transformation of B cells (18). It has been suggested that this may relate to the chronic Ag-driven B cell proliferation and the consequent genetic instability associated with SHM (19). The relationship between the presence of a similar phenomenon in mSG of patients with SS and the development of MALT-L is unknown. Therefore, we retrospectively analyzed the prevalence of GC-LSs in mSG biopsies of 8 SS patients who later developed parotid MALT-L. We showed

that the prevalence of GC-LSs in previous mSG biopsies was significantly increased in these patients compared with the general SS population studied (75% vs 33.3% $p = 0.04$). Although larger and longitudinal studies are needed, this supports the possibility that the presence of GC-LSs in mSG biopsies associated with chronic B cell stimulation may identify a subgroup of patients at an increased risk of developing lymphoma.

Discussion

This study provides evidence for the first time that AID, the enzyme responsible for CSR and SHM of Ig genes, is expressed within ectopic lymphoid tissue of the target organ of a chronic autoimmune disease (the salivary glands of patients with SS), at a level and in a distribution directly comparable to that of secondary lymphoid organs. Because AID expression has been demonstrated to be sufficient for, and exclusively expressed in, B cells undergoing CSR and/or SHM (7), its detection has also enabled us to precisely address the question of whether and where B cells activate their molecular machinery responsible for hypermutation of Ig genes in an ectopic lymphoid tissue.

Importantly, we have shown that within SS-mSGs and SS parotids, AID was invariably expressed in periductal inflammatory foci characterized by features of secondary lymphoid organs such as T/B cell segregation and FDC networks, a phenomenon described as ectopic lymphoid neogenesis (14, 15). Indirect evidence that an Ag-driven clonal proliferation of B cells is taking place in SGs of SS patients was originally provided by Stott et al. (2) who showed a significant level of SHM of the Ig V genes in B cells microdissected from a SS-mSG with FDC networks. Subsequent studies confirmed the selective accumulation of a B cell population characterized by a high rate of mutation in productive rearranged V L chain genes (16, 26). Nonetheless, these studies could not rule out the possibility that already mutated B cells might be recruited from the periphery to the inflamed SG, questioning the effective capability of ectopic GC-LSs to actively support CSR and SHM (16). Our observation of AID expression in all GC-LSs both in mSGs and parotids provides evidence of local activation of the SHM machinery in B cells and strongly supports the notion that these structures provide the microstructural, cellular, and molecular environment to support an Ag-driven T cell-dependent B cell activation and autoantibody production. This possibility is further supported by the evidence that SS patients with AID+ GC in mSG biopsies have an increased prevalence of circulating anti-Ro/La Abs and RF as compared with the general SS population. Further, our data confirm and strengthen the recent observation that anti-Ro/SSA and anti-La/SSB producing cells can be detected in situ within mSGs of SS patients and correlated with serum detection of the same autoantibodies (13). This reinforces the notion that ectopic lymphoid neogenesis in SS is accompanied by local selection and proliferation of autoreactive B cells and that circulating autoantibodies may, at least in part, reflect local production in the salivary glands. In addition, quantitative analysis of AID mRNA in SS-parotids with LESA clearly showed that AID transcripts were strongly expressed at levels comparable or even higher than those observed in lymph nodes. This, together with the high number of ectopic GCs and the evidence of strong AID protein expression, suggests that SS parotids represent a "proper" tertiary lymphoid organ.

A second relevant contribution of this paper is that AID expression in SS-mSGs was not observed in the absence of FDC⁺GC-LSs, suggesting that the presence of CD21⁺FDC networks plays an important role in sustaining local AID expression. Thus, in SS-mSGs, FDC-dependent events such as sustained Ag presentation and/or the provision of growth factors (4) appear to be critical for AID expression allowing B cells to undergo SHM within follicular-like structures. These data would be in apparent contrast with recent reports in rodent secondary lymphoid organs where autoantibody responses have been shown to develop also at extrafollicular sites in the absence of GCs (5, 27), by plasmablasts originating in the T cell-rich red pulp zone of the spleen (28).

Another novel observation of our study is the evidence that, within SS-mSGs and SS-parotids, AID expression identified IF large B cells, a recently described B cell population characterized by dendritic morphology. Interestingly, AID⁺ IF B cells were detected outside B cell follicles but only within SG biopsies characterized by the presence of CD21⁺GC-LSs. This provides the first demonstration that this novel B cell population can be found in an ectopic lymphoid tissue. So far, IF large B cells were described only in lymph nodes mainly localized within the interfollicular T cell-rich areas (8). Recent reports also suggested that these cells have undergone CSR and/or SHM and represent the only B cell subset expressing detectable

AID outside GCs in secondary lymphoid organs (8–10). The general function of IF large B cells and the significance of sustained AID expression in this cell type outside FDC networks are currently unknown. The prevalent cytoplasmic expression of AID in IF large B cells, with a lower prevalence of nuclear AID staining is in line with previous reports in secondary lymphoid organs where cytoplasmic AID accounts for >90% of total AID within GC B cells (29) and large IF B cells (9, 10). Accordingly, it has been shown that in B cells AID is predominantly located in the cytoplasm due to an active export system from the nucleus (30).

Finally, in this report we demonstrated that in SS parotid low-grade MALT-Ls, AID expression is not detectable within the malignant MZB cell population, confirming similar findings in MALT-Ls, mostly of non-SG origin, recently reported while preparing this manuscript (31). Conversely, AID was selectively expressed by GC B cells and IF large B cells within the residual reactive component of the MALT-L characterized by CD21⁺FDC networks. Accordingly, in SS-MALT-L, AID mRNA closely associated with CD21L, an isoform of CR2/CD21 selectively expressed by FDC(24) supporting the evidence that AID expression in lymphomatous parotids is still dependent on FDCs. This explains the apparent paradox of strong AID mRNA expression in MALT-L and absent protein expression in malignant MZB cells recently reported (31). Lymphomas in SS are most commonly non-Hodgkin B cell lymphomas of the MALT, originating from MZB cells (17, 32). Although a single differentiation marker between the reactive and the malignant B cell populations within MALT-L is not available, we could identify halos or sheets of malignant monocytoid B lymphocytes with pale nuclei and infiltrating ductal structures with a CD20⁺/IgD⁻/bcl-2⁺ MZ-like phenotype (17, 32, 33). This population was easily differentiated from the CD20⁺/IgD⁺/bcl-2⁺/bcl-6⁻ population of FoB and the CD20⁺/IgD⁻/bcl-2⁻/bcl-6⁺ GC B cells associated with CD21⁺FDC⁺ residual GC that was referred to as the "reactive" component of the MALT-L. Interestingly, residual reactive areas with numerous ectopic GCs were detected in all parotid SS-MALT-L supporting the possibility that the Ag-driven T cell-dependent B cell activation within ectopic lymphoid tissue may support a process of B cell clonal selection and escape of malignant clones. This would be in keeping with the recent demonstration that a common clonal lineage exists between the polyclonal and later monoclonal B cell population with progression from LESA toward SG and extraglandular MALT-Ls (18, 34) indicating a multistep Ag-driven process within SGs. The absence of AID expression in CD20⁺/IgD⁻/bcl-2⁺ MZB cells is also in keeping with recent reports demonstrating absence of mRNA and protein expression of AID in MZB cells of mice and humans (35, 36). Because normal splenic MZ display somatically mutated V genes (37, 38) the lack of detectable AID expression led to the suggestion that Ig-mutated MZB cells derive from precursors which have undergone SHM within GCs (35). In this regard, demonstration of the absence of AID within neoplastic MZB cells in SS MALT-L is very relevant because malignant MZB cells display highly mutated IgV H and L chain genes both in gastric and SG MALT-Ls (20, 21, 39, 40). In addition, it has been suggested that SHM may even be ongoing within the clonal population of low-grade gastric and SG MALT-Ls as demonstrated by intraclonal variation (20, 21), a result which led to the knowledge that malignant B cells in MALT-Ls express a post-GC phenotype (20, 21, 39, 40). The demonstration

of the absence of AID in the malignant MZ-like B cell population within SS-MALT-Ls suggests that the intracлонаl diversification may occur within FDC networks, possibly as a result of an Ag-driven T cell-dependent stimulation. In support of this possibility, it has been demonstrated that in gastric MALT-Ls malignant MZB cells proliferation is critically dependent on *Helicobacter pylori*-specific T cells (41). In turn, outside GCs, clonally related MZB cells may undergo proliferation without reactivation of the hypermutation process and depend on local triggering for survival. In this regard, recent reports demonstrated that SG MALT-Ls, most from SS patients, often display IgV_H-CDR3 with RF homology implying that a percentage of SS-MALT-Ls originate from precursors that bear a functional autoreactive BCR (42, 43). Interestingly, studies in rodents showed that RF producing cells can be activated by dual engagement of BCR and TLR9 by IgG2a-chromatin immune complexes (44). Whether integrated signaling through BCR and TLRs may trigger proliferation of mutated malignant B cells in human parotid MALT-L remains to be demonstrated.

In conclusion in this study we provide original evidence that in SGs of SS, AID is exclusively and invariably expressed in the presence of FDC networks and identifies IF large B cells. In addition, we show that neoplastic MZB cells do not express detectable AID, as its expression is confined to reactive areas within SS-MALT-Ls. These results strongly support the notion that ectopic lymphoid structures in SS-SGs sustain local Ab production and suggest their implication in lymphoma development.

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Disclosures

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