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Ig Gene Analysis Reveals Altered Selective Pressures on Ig-Producing Cells in Parotid Glands of Primary Sjögren's Syndrome Patients

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In this study, we sought to understand the selective pressures shaping the Ig-producing cell repertoire in the parotid glands of primary Sjögren's syndrome (pSS) patients before and after rituximab treatment (RTX). In particular, we evaluated the role of potential *N*-glycosylation motifs acquired by somatic hypermutation (ac-Nglycs) within Ig H chain V region (IGHV) genes as alternative selective pressures for B cells in pSS. Five pSS patients received RTX. Sequential parotid salivary gland biopsies were taken before RTX, at 12 wk and at 36–52 wk after treatment. Parotid biopsies from four non-pSS patients served as controls. Sequence analysis was carried out on the IgA and IgG RNA transcripts expressing IGHV3 genes in all parotid biopsies. Both IgG and IgA sequences from pSS patients exhibited no evidence for positive Ag-driven selection pressure in their CDRs in contrast to non-pSS controls. The prevalence of IgG sequences with ac-Nglycs was significantly higher in pSS patients than in non-pSS controls. Selection pressures shaping the IgG and IgA repertoire within pSS patients' parotid glands are distinct from those in non-pSS controls, with very little evidence for positive (auto)antigen selection. The higher prevalence of ac-Nglycs on pSS-IgG compared with non-pSS IgG indicates that ac-Nglycs could be an alternative form of selection pressure. We speculate that B cell hyperproliferation within parotid glands of pSS patients may result from Ag-independent interactions such as that between glycosylated B cell receptors and lectins within the microenvironment rather than (auto)antigen-specific stimulation. Our study brings a new perspective into research on pSS. ***The Journal of Immunology*****, 2015, 194: 514–521.**

Sjögren's syndrome (SS) is an autoimmune disorder that primarily affects the salivary and lacrimal glands. The main clinical features are progressive dryness of mouth and eyes. When these symptoms occur without the presence of another systemic autoimmune disease, it is called primary SS (pSS) (1).

A hallmark of pSS is the presence of lymphocytic infiltrates composed of T cells, B cells, and plasma cells in the salivary glands (2), and the infiltrates can even be organized as ectopic lymphoid tissue, sometimes containing germinal center–like structures (3). Disturbances in relative proportions of peripheral B cell subsets and high titers of circulating autoantibodies such as anti-Ro (SS-A) and anti-La (SS-B) in the blood are key features of pSS (4). There is also an increased risk for the development of B cell malignancies in pSS patients (3, 5). Furthermore, therapeutic B cell depletion strategies targeting CD20 provide clinical relief from pSS symptoms (6, 7). However, symptoms usually return 6–9 mo after treatment, and disease relapse apparently coincides with the reappearance of peripheral B cell subpopulations in the blood (7, 8). All of the above observations strongly suggest that B cells and/or plasma cells play a significant role in the disease mechanisms underlying pSS, although their exact role in pSS pathogenesis is still undefined.

Selection of B cells occurs during normal humoral immune responses. During T cell–dependent immune responses, activated B cells divide rapidly in germinal centers and mutations are introduced into their Ig V region gene segments by somatic hypermutation. These hypermutations affect the ability of B cell receptors to bind Ags and, consequently, B cells are selected based on their affinity to Ags. This results in the affinity maturation of the Ab response. The analysis of replacement and silent mutational frequencies in the CDRs that contact Ag and the framework regions (FRs) that encode structural integrity can identify or predict Ag selection patterns (9). This led to the creation of multiple computational tools that predicted Ag selection pressures using statistics and known mutational patterns (10, 11). In this study, we used a program called BASELINe (12) to analyze the distribution of replacement versus silent mutations and determine the different selection pressures shaping the Ig repertoire.

The consequences of somatic hypermutations are not limited to altered Ag-binding properties, but may also affect amino acid composition that influences other binding properties. Previous

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Abbreviations used in this article: ac-Nglyc, *N*-glycosylation motif acquired by somatic hypermutation; CI, confidence interval; FR, framework region; IGHV, Ig H chain V region; Nglyc, *N*-glycosylation motif; pSS, primary SS; RTX, rituximab treatment; SpA, staphylococcal protein A; SS, Sjögren's syndrome.

studies reported that *N*-glycosylation motifs (Nglycs) created or acquired after somatic hypermutation could play a role in conferring a selective advantage to B cells (13–17). This led us to evaluate the contribution of acquired Nglycs after somatic hypermutation within IGHV genes toward the selection of B cells in pSS.

We previously reported the persistence of Ig-producing cells in parotid salivary glands of pSS patients despite B cell depletion with rituximab treatment (RTX) (18). One reason for this could be the lack of CD20 expression on plasma cells. However, there are reports of $CD20^+$ plasma cells being present in tonsils even after RTX (19). We also observed more mutations in the Ig H chain V region (IGHV) genes obtained from salivary glands after RTX compared with their clonally related counterparts present before RTX (18). This is an indication that Ig-producing cells surviving after RTX continue to proliferate. However, the factors promoting selection and survival of these Ig-producing cells are still unclear.

Hence, a major objective of our study was to analyze mutation patterns within IGHV gene sequences derived from pSS salivary glands. Through this approach, we gained an overall insight into the type and extent of selection pressures influencing the B cell repertoires in the parotid glands of pSS patients before and after RTX. In particular, we evaluated the role of acquired *N*-glycosylation motifs in the selection of Ig-producing cells in pSS and their survival after RTX.

Materials and Methods

Patients

Five pSS patients (all females; mean age, 49.5 y; range, 36–65 y) with disease duration of <5 y were enrolled in this study after signing an informed consent. These patients fulfilled the 2002 American–European criteria (20) and the recently published American College of Rheumatology criteria for pSS (21). Inclusion criteria of the pSS patients used in this study were stimulated whole saliva secretion flow of >0.15 ml/min, presence of autoantibodies (IgM rheumatoid factor of ≥10 kIU/l in combination with anti–SS-A and/or anti-SS-B autoantibodies), and a salivary parotid gland biopsy (obtained ≤12 mo before inclusion) showing characteristic features for SS (22). The five pSS patients were treated with RTX as described before (7). All patients showed significant depletion of B cells in the peripheral blood at 12 wk after RTX. Incisional biopsies of the parotid gland were obtained from the same gland before RTX and at 12 and 36–52 wk after RTX by the same surgeon (F.K.L.S.) under local anesthesia (23). The biopsies were frozen in liquid nitrogen immediately after surgery and stored at −80°C.

As non-pSS controls, two patients with sicca complaints not fulfilling the American–European and American College of Rheumatology criteria for pSS and two patients with malignancies (squamous cell carcinoma of the oral cavity) without involvement of the parotid salivary glands were included. From these four non-pSS controls, we obtained single parotid biopsies with laboratory-confirmed normal histology. The study protocol was approved by the Institutional Review Board of the University Medical Center Groningen.

Cloning, sequencing, and analysis of Ig sequences

Parotid gland (diagnostic) biopsies are quite small. Hence, we prudently maximized the use of these biopsies by conducting our study on a few tissue sections from each biopsy and not on whole biopsies. The rest of the sections were used for immunohistochemistry in our previous study (18).

Essentially, three serial batches of four tissue sections (5–7 mm thick) were obtained from different parts of each biopsy. Total RNA was extracted from each batch. These RNA samples were converted to cDNA and amplified by RT-PCR using primers specific for the IGHV3 gene (24) in combination with C region primers specific for the CH1 domains of the Cα (25) or Cγ (18) C regions. RT-PCR and cloning were performed as reported previously (18). A total of 72 (36 IgA and 36 IgG) plasmid constructs were picked separately for each biopsy (after checking for RT-PCR product insertion) and submitted for sequencing.

The IGHV regions and isotype subclasses were identified using ImMunoGeneTics databases (26). Identical/similar sequences (different by ≤2 mutations to account for Taq error rate) derived from the same PCR were considered redundant and counted as one. Only productive sequences (encoding functional proteins) were included in this study. Ig-producing cells were considered as clonally related (hereafter referred to as clones) based on their similarity of nucleotide sequence at the CDR3, their shared IGHV-D-J gene usage, identical/similar N1 and N2 additions, and their pattern of shared mutations within Ig V regions (27). All sequences were submitted to GenBank for approval.

Analysis of selection pressures

Selection pressure analysis was carried out with the BASELINe program (Bayesian estimation of Ag-driven selection) (12). BASELINe provides a visually quantitative method to analyze selection pressures exhibited by Ig sequences on the basis of mutation patterns. BASELINe's key features are its ability to aggregate selection strengths of different sequences within an experimental group and statistically compare selection pressures of different experimental groups (12). Sequences that are unmutated or contain insertions and/or deletions are excluded by BASELINe. Table I indicates sequence numbers analyzed by BASELINe in this study.

Prediction of potential N*-glycosylation motifs*

Prediction of potential Nglycs was carried out on translated sequences of IGHV-D-J regions (using ImMunoGeneTic's HighV-QUEST program). Nglycs were predicted using the NetNGlyc1.0 online tool (http://www.cbs.dtu.dk/services/NetNGlyc/). *N*-glycosylation is known to occur on asparagines (N), which occur within an N-X-serine (S)/threonine (T) motif (where X is any amino acid except proline, as it precludes *N*-glycosylation owing to steric hindrance). We also excluded the motif N-X-S when tryptophan, aspartic acid, glutamic acid, and leucine were at position X, as these are poor oligosaccharide acceptor motifs (28). The criteria set for accepting prediction of Nglycs were potential >0.5 and jury agreement ≥5 of 9 (29). For Nglyc prediction, we analyzed all sequences, including clonal sequences, as several clones had members of different isotypes (Table I). Most Nglycs (93 of 95) detected in this study were results of somatic mutations (acquired motifs), and only these were considered because we wanted to evaluate the contribution of somatic hypermutation to B cell selection.

Statistical analysis

The numbers of Nglycs in different groups were statistically compared using the χ^2 test. Because the sequence numbers analyzed varied within groups, we used the Freeman–Tukey arcsine transformation method for the meta-analysis of proportions to estimate the pooled proportion. The meta-proportions or frequencies are indicated with 95% confidence intervals (CI).

Results

A total of 543 productive Ig sequences was obtained from five pSS patients, before and after RTX, and 93 productive Ig sequences were obtained from four non-pSS controls (Table I). The nucleotide sequences obtained in this study, which were ≥200 bp, were submitted to GenBank (accession IDs KP091906–KP092227 and KP092228–KP092520; http://www.ncbi.nlm.nih.gov/genbank). The complete set of sequences will be available on request. In the five pSS patients, we observed a total of 23 clones whose members were present both before and after treatment. Of these 23 clones, two clones were composed of only IgA sequences, whereas 14 clones had only IgG sequences and five clones were made up of IgG plus IgA sequences. In the four non-pSS controls, we observed only a single clone with two members belonging to the IgA isotype.

Although we picked >1000 plasmid constructs with Ig sequences, >300 constructs were discarded after sequencing due to poor sequence quality or because they contained unidentifiable (after comparison with GenBank) sequences. Unproductive or redundant sequences (identical sequences from the same PCR) as well as non-Ig sequences and IGHV sequences lacking a fully designated V-D-J rearrangement (as per ImMunoGeneTic's HighV-QUEST) were also eliminated from our analyses. In addition to this, clonal sequences with ≤2 unshared mutations (to account for Taq error rate) were excluded when they expressed identical isotypes and were from the same PCR.

Table I. Number of Ig sequences analyzed in this study

Sample Group	No. of IgA Sequences	No. of IgG Sequences	Total No. of Sequences
Non-pSS controls (n = 4)	45 (42)	48 (44)	93 (86)
pSS (before RTX) (n = 5)	132 (123)	97 (87)	229 (210)
pSS (RTX plus 12 wk) (n = 5)	112 (107)	72 (72)	184 (179)
pSS (RTX plus 36-52 wk) (n = 5)	72 (62)	58 (58)	130 (120)

The number of IgG and IgA sequences obtained in this study from non-pSS controls and from pSS patients at baseline, at 12 wk after RTX, and at 36–52 wk after RTX are shown. A total of 543 productive IGHV3 Ig sequences were obtained from five pSS patients. The numbers of sequences that were analyzed by the BASELINe software are indicated in parentheses. Sequences that were unmutated or contain insertions and/or deletions were excluded by BASELINe.

Hence, out of 938 readable sequences obtained in this study, 63 (6.7%) were unproductive, 225 (24%) were redundant, and 107 (11.4%) sequences were either non-Ig or lacked a complete V-D-J rearrangement.

Prediction of Ag selection pressures

In the BASELINe program, positive selection pressure indicates an increased presence of mutations that results in nonconservative amino acid changes, and such patterns are usually seen in the case of B cells influenced by Ag selection. Alternatively, negative selection pressure implies conservation of protein structure where mutations result in identical or similar (conservative) amino acid changes (12). BASELINe also shows relative differences in selection pressures between experimental groups using color codes. Increasing shades of red indicate the shift toward more positive selection pressure, whereas different green shades indicate shifts toward negative selection pressures (Figs. 1, 2).

Both IgG and IgA sequences from non-pSS controls (non-pSS IgG and non-pSS IgA, respectively) exhibited positive selection pressures in their CDRs and negative selection pressures in their FRs (Figs. 1, 2). This is consistent with the idea that CDRs are the main Ag-binding regions, whereas FRs are generally conserved regions that preserve the structural integrity of Igs. However, in pSS patients, both IgG and IgA sequences (pSS-IgG_1 and pSS-IgA_1, respectively) exhibited negative selection pressures in their CDRs, despite being mutated. Compared to non-pSS controls, both pSS-IgG and pSS-IgA showed significantly greater negative selection pressures in their CDRs and FRs (Figs. 1, 2).

When pSS-IgG before and after RTX were compared, no significant changes in selection pressures were noted in FRs or CDRs (Fig. 1). However, at 12 wk after RTX (indicated as pSS-IgG_2) and at 36–52 wk after RTX (indicated as pSS-IgG_3), the selection pressures on the CDRs were still significantly different compared with the CDRs of non-pSS IgG (p = 0.04 and p = 0.004, respectively). The strong negative selection pressures exhibited by pSS-IgG FRs before RTX remained significantly greater at both time points after RTX when compared with non-pSS IgG FRs. This suggests the persistence of certain subpopulations of IgG-producing cells.

The IgA sequences analyzed before and after RTX showed the same pattern as the IgG sequences. At 12 wk after RTX, the IgA sequences (indicated as pSS-IgA_2) analyzed exhibited selection pressures in their CDRs that were not significantly different from non-pSS IgA. However, at 36–52 wk after RTX (indicated as pSS-IgA_3) the selection pressures on CDRs were again significantly different compared with both non-pSS IgA (p = 0.002) and also different compared with pSS-IgA_2 (p = 0.0134). The stronger negative selection pressures in FRs of pSS-IgA compared with non-pSS IgA as seen before RTX also were not present at 12 wk after RTX and at 36–52 wk after RTX, as these selection pressures returned to the patterns observed in pSS-IgA before RTX (Fig. 2).

In conclusion, the Ag-driven selection pressures that are seen in the B cell repertoire as a result of humoral immune responses in non-pSS controls are significantly different from those seen in salivary glands from pSS patients. Administering RTX to pSS patients temporarily results in selection pressures that are similar to those seen in non-pSS controls. However, with time, the selection pressures relapse to characteristic pSS patterns that were observed before RTX.

Incidence of N*-glycosylation sites in IGHV3 regions*

Using the BASELINe tool, we observed no evidence for Ag-driven positive selection pressure in the CDRs of pSS-IgG and pSS-IgA, as opposed to non-pSS control sequences. This suggests that factors other than classical Ag-driven selection may contribute to the selection of Ig-producing cells in pSS patients. We evaluated whether *N*-glycosylation could be one of these factors because the acquisition of potential *N*-glycosylation sites by somatic hypermutation was shown to confer a selective advantage to certain B cells (13). Most Nglycs (93 of 95) detected in this study were results of somatic mutations (acquired motifs), and only these were considered because we wanted to evaluate the contribution of somatic hypermutation to B cell selection.

The total number of *N*-glycosylation motifs acquired by somatic hypermutation (ac-Nglycs) observed in Ig sequences from pSS patients before RTX (44 of 229 sequences) was significantly higher (p = 0.005; χ^2 test) than Ig sequences from non-pSS controls (9 of 93 sequences) (Table II). When we took into account the differences in sample sizes within each group, we observed that the prevalence of ac-Nglycs was significantly higher in pSS-IgG before RTX (Fig. 3; p = 0.001; 24.1%; CI, 16.3–32.8) compared with non-pSS IgG (5.8%; CI, 1.1–13.7). Compared to before RTX, the prevalence of ac-Nglycs in pSS-IgG decreased significantly at 12 wk after RTX (p = 0.04). However, at 36–52 wk after RTX, the prevalence was similar to that seen before RTX.

The prevalence of ac-Nglycs was not different between pSS-IgA before RTX and non-pSS IgA (p = 0.5; Fisher exact test), and no change was seen among pSS-IgA after RTX.

To further differentiate between acquisition of Nglycs in pSS patients and non-pSS controls, we analyzed the localizations of these ac-Nglyc motifs within IGHV genes (Table II). The prevalence of ac-Nglycs in FRs of sequences from pSS patients before RTX was significantly higher than that seen in non-pSS patients (p = 0.001). This prevalence was not affected by RTX.

Among the 44 Ig sequences with ac-Nglycs from pSS patients before RTX, 26 of 44 sequences (~60%) had sites in the FR3 region (11 from IgA and 15 from IgG), although the prevalence of FR3-associated ac-Nglycs was only significantly different between pSS patients and non-pSS controls among IgG sequences (p = 0.020) and not among IgA sequences. Of these 26 sites noted in FR3s in pSS patients before RTX, 19 (76%) sites were created by mutations at residue 84, and the remaining mutations were at residues 77 and 93 (three sequences each). Seven of 26 of these FR3 *N*-glycosylated sequences belonged to five different clones with members present both before and after RTX.

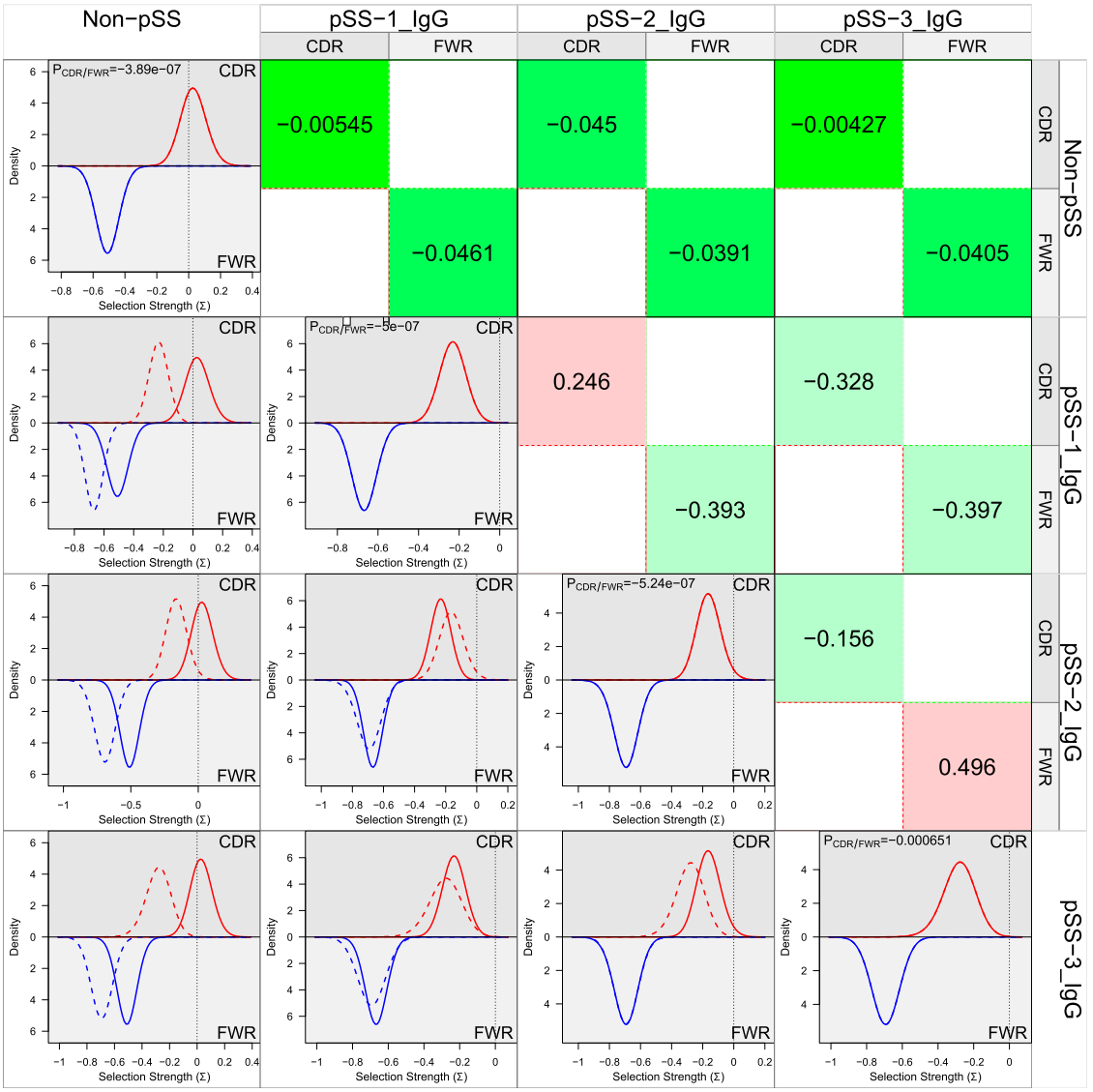

FIGURE 1. Selection pressures on IgG-producing cells in non-pSS and pSS patients. The panel shows selection pressures exhibited by IgG sequences from four non-pSS controls (Non-pSS IgG) and five pSS patients before RTX (pSS-IgG_1) at 12 wk after RTX (pSS-IgG_2) and at 36–52 wk after RTX (pSS-IgG_3). All figures were obtained as output data from the BASELINe program. Comparison of selection pressures is shown in a two-point table format with the differences in selection pressures represented using both probability distribution graphs and statistically derived numerical values. Comparisons are carried out between a group in a particular column over that in the corresponding row. Within graphs showing the distribution graphs of two groups, the solid line represents the column group whereas the dotted line represents the row group. Negative sign and green color indicate negative selection pressures, whereas red color indicates positive selection. The strength of relative selection pressures is also indicated with varying intensities of the color codes. The p values < 0.05 indicate significant differences based on the binomial statistical test incorporated in BASELINe.

Discussion

Our results suggest that the selection pressures shaping the IgG and IgA repertoire within pSS patients' parotid glands are distinct from those in non-pSS controls. We also noted a higher prevalence of ac-Nglycs in pSS-IgG before RTX compared with non-pSS IgG, which could indicate an additional form of selection pressure on these IgG-producing cells.

Emphasis on conservation of Ig structural integrity rather than on Ag selection in pSS patients

The distribution of replacement/silent mutations is used by BASELINe to investigate the role of Ag selection in optimizing the Ig repertoire (12). The suitability of using BASELINe for evaluating selection pressures was effectively demonstrated by a previous study that used the BASELINe algorithm in its prototype version called the Focused test (11) on Ig sequences from Ag-specific B cells during primary and memory immune responses in immunized mice (30). In this previous study (30), the BASELINe/Focused test algorithm was able to clearly detect positive selection within the CDRs and FRs of memory B cells from wild-type mice as well as negative selection in FRs of memory B cells from mice.

An important observation in our study was that despite being highly mutated, both IgG and IgA sequences from pSS patients showed no positive selection pressures in their CDRs. We also observed an increased emphasis on the maintenance of structural integrity (i.e., increased negative selection pressures) in the FRs of pSS-IgG and pSS-IgA compared with non-pSS controls. This is in line with earlier observations that showed altered selection in different autoimmune diseases, including pSS (31).

These observations suggest that activated B cells that become Ig-producing cells in pSS patients may be selected by unconventional processes that are probably not (auto)antigen-driven. It is also

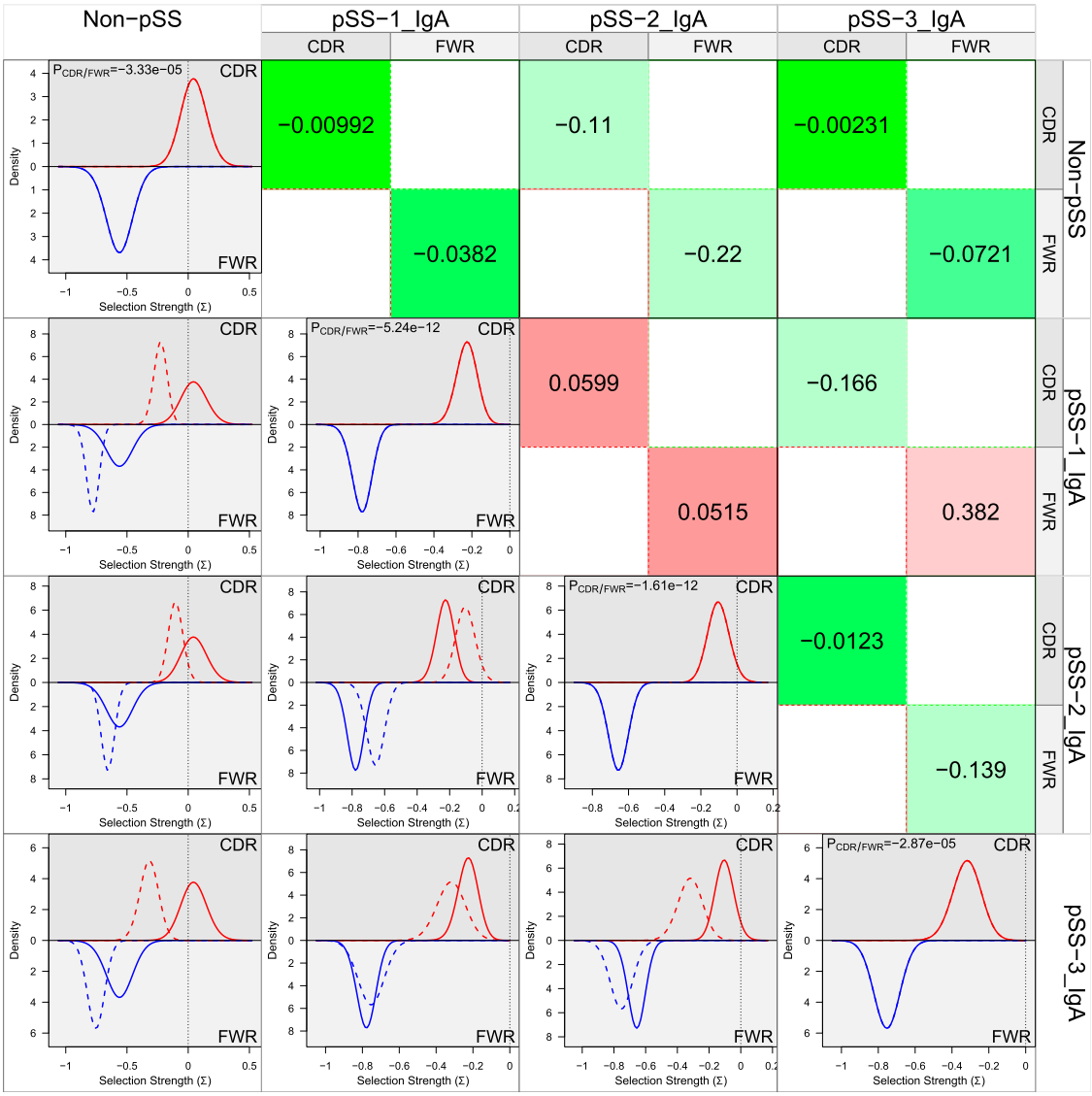

FIGURE 2. Selection pressures on IgA-producing cells in non-pSS and pSS patients. The panel shows selection pressures exhibited by IgA sequences from four non-pSS controls (Non-pSS IgA) and five pSS patients before RTX (pSS-IgA_1) at 12 wk after RTX (and pSS-IgA_2) and at 36–52 wk after RTX (pSS-IgA_3). All figures were obtained as output data from the BASELINe program. Comparison of selection pressures is shown in a two-point table format with the differences in selection pressures represented using both probability distribution graphs and statistically derived numerical values. Comparisons are carried out between a group in a particular column over that in the corresponding row. Within graphs showing the distribution graphs of two groups, the solid line represents the column group whereas the dotted line represents the row group. Negative sign and green color indicate negative selection pressures, whereas red color indicates positive selection. The strength of relative selection pressures is also indicated with varying intensities of the color codes. The p values < 0.05 indicate significant differences based on the binomial statistical test incorporated in BASELINe.

possible that such processes may occur outside classical germinal centers (32, 33).

We noted that the strong negative selection pressures exhibited by pSS-IgG FRs before RTX remained significantly greater at both time points after RTX when compared with non-pSS IgG FRs. This may be an indication of the persistence of certain subpopulations of IgG-producing cells in the wake of B cell depletion by RTX.

At 12 wk after RTX, there was a temporary decrease in pSS-IgG and pSS-IgA sequences exhibiting selection pressures characteristic of pSS patients before RTX. This is a period when pSS patients experience clinical relief following RTX. This is also the time point when patients exhibit marked B cell depletion in their peripheral blood (8). Then, at 36–52 wk after RTX, the selection pressures of both pSS-IgG and pSS-IgA revert to the patterns seen before RTX. We speculate that the recurrence of selection pressures characteristic of pSS repertoires is probably due to the reappearance of Ig-producing cells that are clonally related (18) or similar to the cell populations persisting even after RTX.

It is conceivable that because we extracted mRNA from tissue sections and did not select for specific B cell subsets, most Ig sequences obtained could be from class-switched plasmablasts or plasma cells, which reportedly express ~100- to 1000-fold more Ig transcripts compared with B cells (34). Alternatively, this approach may provide a greater representation of Ig-producing cells located within glandular tissues of pSS patients that are active producers of pathologically significant levels of autoantibodies such as anti-SSA/Ro and anti-SSB/La Abs (35).

Acquisition of N*-glycosylation motifs on FRs of IgG-producing cells in pSS patients may contribute to their selection*

Our study presented minimal evidence for positive Ag selection pressure on IGHV sequences from B cells in the parotid glands of

Table II. Acquired *N*-glycosylation sites and motifs observed in Ig sequences

Sample Group	Patient	FR1	CDR1	FR2	CDR2	FR3	CDR3
Non-pSS controls	NC-1		**NFS**				
	NC-2				**NIT**		
	NC-3		NSS, NSS			NNT	NVT, NRT
	NC-4		NFS			NKS	
pSS (before RTX)	pSS-1		NIS, NVS			**NDT**, NGS, **NNT**, NIS, NHT, **NNT**, NIS, NNT, NKT, **NGS**, NGT	NIS, NGT
	pSS-2		NSS			**NNT**, **NNT**	NVT
	pSS-3		**NSS**, NYS			**NNT**, NNT, **NNT**, **NNS**, **NIS**, **NLT**, **NNT**	**NGT**
	pSS-4		NFT, **NFS**		NGT, **NYT**	**NYS**, **NYS**, NTT	**NNT**
	pSS-5		NYS, NFS	**NIS**	**NTT**, **NTS**	NDT, NDT, **NNT**	
pSS (RTX plus 12 wk)	pSS-1		NFS, **NIS**	NNT		NNT, **NST**, NNT, NNT, NNT,	NGS
	pSS-2				**NGT**, NGT	**NNT**	
	pSS-3					**NNT**, **NNT**	
	pSS-4		NVS, NFS			**NTT**, **NNT**, NFS	**NGT**
	pSS-5		NVS, NFT			NDT, NDT, NNS, **NNT**	NAT, NYS
pSS (RTX plus 36–52 wk)	pSS-1		**NRT**				
	pSS-2						
	pSS-3				NRT		
	pSS-4		NIS, **NVT**			**NNT**, **NTT**, **NNT**, **NNS**, **NYT**	NGT
	pSS-5	NTS	NFS			NVT, **NDT**	

The amino acid motifs of acquired *N*-glycosylation sites in Ig sequences from different individuals included in this study are shown here along with their localization in CDRs or FWRs. Ac-Nglycs observed in IgG sequences are indicated in bold letters. Amino acids are designated with standard codes.

NC, non-pSS controls.

pSS patients when compared with non-pSS controls. This raises the possibility that the hyperproliferation of B cells in pSS patients may not be triggered by (auto)antigen-specific stimulation. We speculate that in pSS patients, Ig sequences may confer a selective advantage on their B cells by encoding for certain properties or posttranslational modifications on the BCRs rather than through the specificity of Ab–(auto)antigen interactions. In this regard, studies of ac-Nglycs within Ig V regions in B cell malignancies provided interesting clues as to a potential role for the posttranslational modification of *N*-glycosylation in conferring a selective advantage to B cells (13–17).

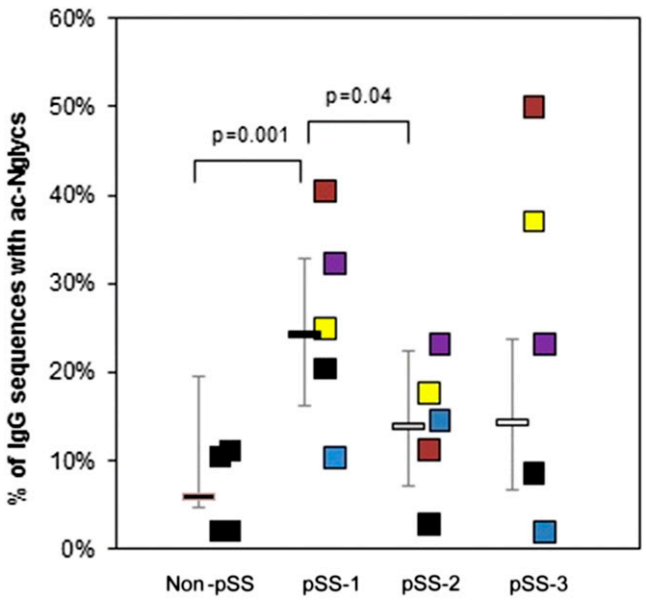

FIGURE 3. Increased prevalence of acquired *N*-glycosylation motifs in IgG sequences from pSS patients. The prevalence of ac-Nglycs in IgG sequences are compared between four non-pSS controls and five pSS patients before RTX (pSS-1) using the Freeman–Tukey arcsine transformation method. The five pSS patients have been differentiated using color codes. The frequencies of ac-Nglycs in IgG sequences from pSS patients were also analyzed at 12 (pSS-2) and 36–52 (pSS-3) wk after RTX. Statistically significant differences are indicated by *p* values.

During the modulation of the Ig repertoire, the somatic hypermutation process introduces mutations in the V regions of both H and L chains that either create new *N*-glycosylation (acquired) sites or destroy such sites already present in germline variable genes (36). We observed a significantly higher prevalence of IgG sequences with ac-Nglycs in their IGHV regions in pSS patients compared with non-pSS controls. This suggests that some IgG-producing cells in the parotid glands of pSS patients may have been selected on the basis of their glycosylated BCR.

The role of glycosylated BCRs in B cell activation through binding to lectins has been reported previously (37). Lectins are not only expressed on the surface of pathogens, but also on components of the microenvironment, such as T and NK cells (38, 39) as well as macrophages and dendritic cells (40). Interestingly, lectins are also present on acinar and ductal cells of the secretory ducts within salivary glands (41), which are the primary sites of pathology in pSS. We speculate that glycosylated BCR interactions with lectins in the parotid gland microenvironment may drive B cell proliferation and ongoing somatic hypermutation in pSS patients (18) in a non-classical way, independent of (auto)antigen-binding specificity (37).

Furthermore, the increased acquisition of potential Nglycs noted within pSS-IgG sequences was not observed among pSS-IgA, indicating that the IgG constant region may play an as yet undefined but vital role in the selection and survival of IgG-producing cells with glycosylated BCRs in pSS patients. An interesting study by Berkowska et al. (42) showed that human memory B cells can originate from three distinct germinal center–dependent and –independent maturation pathways. In that study, the authors gave evidence for all IgG responses and certain IgA responses as being germinal center–dependent. Their studies also indicated the existence of germinal center–independent IgA responses.

Acquired Nglycs were found among both IgG and IgA sequences. However, their incidence was significantly increased in pSS-IgG only and not in pSS-IgA compared with non-pSS. This suggests that the selection of B cells expressing different isotypes may occur through different mechanisms and may perhaps even depend on the microenvironment within diseased glands, as in-

dicated by the study by Berkowska et al. (42). It could be that IgG-producing B cells with ac-Nglycs are able to selectively interact more with lectins within germinal centers in the diseased glands than IgA-producing B cells with ac-Nglycs.

At the same time, Fig. 2 clearly shows that the selection pressures reflected in the BASELINe results of pSS-IgA sequences both before and after RTX (at relapse) are significantly different from those in non-pSS IgA. We think that this is an indication of the existence of other selection pressures besides ac-Nglycs. By analyzing the incidence of ac-Nglycs and their association with IgG, we think we have narrowed in on one of these selection pressures. IgG- and IgA-producing B cells without ac-Nglycs in pSS diseased glands may be selected in other ways that are as yet unclear.

We also noted that in pSS patients, most (60%) of these ac-Nglycs were situated within the FR3 regions. Interestingly, FR3 domains are also associated with B cell activation through their interactions with superantigens. Superantigens such as the staphylococcal protein A (SpA), the endogenous human gut-associated sialoprotein pFv, and the HIV-1 envelope protein gp120 interact with the evolutionarily conserved IGHV framework regions and stimulate B cell differentiation and Ig secretion (43). SpA interacts specifically with IGHV3 sequences between residues 75 and 84 of the FR3 region (44). The gp120 and pFv proteins also share a similar affinity to the same or nearby FR3 sites, as was shown by competitive binding experiments with SpA (43, 45). Such unique interactions between superantigens and FRs are remarkable indications of how interactions within FRs, and particularly FR3, can result in B cell activation, independent of conventional Ag binding at the CDRs. This concept is significant because the superantigen-interacting FR3 sites targeted by SpA, gp120, and Fv are at the same locations where we observed nearly 60% of all *N*-glycosylation motifs (residues 77, 84, and 93) occurring within IGHV sequences from pSS patients.

Our observations suggest that the causes behind the B cell hyperproliferation seen in pSS and perhaps other autoimmune diseases in general may go beyond the traditionally accepted theory of autoantigenic stimulation. Our data are in line with previously published studies that suggested a role of glycosylated IgG BCRs in pSS and other autoimmune diseases (46–48). However, these studies mainly focused on Fc region glycosylation and did not analyze the altered glycosylation pattern in Fab regions introduced by the somatic hypermutation process. Another study (49) that took both Fab and Fc glycosylation patterns in pSS patients into consideration reported that the proportion of sialylated IgA1 and IgA2 was increased in pSS patients compared with normal controls. This was postulated to decrease binding of IgA by immune receptors and result in altered clearance (50). We are currently evaluating as to whether our observations in pSS could be relevant in the context of other autoimmune diseases as well.

One limitation of our study might be that we were only able to analyze the glycosylation patterns among members of the V_H3 gene family. This was due to the dominance of the expression of the V_H3 gene family genes within our relatively small biopsy samples. In a recent study we used deep-sequencing technology (also known as next-generation sequencing) to analyze all Ig transcripts from all V_H gene families in samples from five untreated pSS patients and five non-pSS patients with sicca complaints. The next-generation sequencing data indicate that most dominant clones now obtained by unbiased deep sequencing belong to the V_H3 gene family. Other dominant clones expressed V_H4 gene family genes and a minority expressed V_H5 genes. This explains why in our present experimental study using limited parotid tissue sections, non-V_H3 gene families were rarely detected. More importantly, these new data indicated that in these dominant clones the percentage of Ig sequences with acquired Nglycs is increased in (untreated) pSS patients compared with non-pSS sicca controls, and that this increase is not restricted to V_H3 gene sequences but could also be observed in V_H4 gene sequences (A. Visser et al., manuscript in preparation).

To conclude, selection pressures shaping the IgG and IgA repertoire within pSS patients' parotid glands are distinct from those in non-pSS controls, with very little evidence for positive Ag selection. This suggests that factors other than classical (auto) antigen-driven selection may contribute to selection of certain Ig-producing cells in pSS patients. We evaluated whether ac-Nglycs could be one of these factors and found a higher prevalence of ac-Nglycs in pSS-IgG before RTX compared with non-pSS IgG. This indicates that ac-Nglycs could be an alternative form of selection pressure. Our observations lead us to speculate that B cell hyperproliferation within the diseased glands of pSS patients may be the result of Ag-independent interactions such as that between ac-Nglycs and lectins within the microenvironment.

Disclosures

The authors have no financial conflicts of interest.

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