T cell-dependent B cell hyperactivity in primary Sjögren's syndrome
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GENE EXPRESSION PROFILING OF EPITHELIUM-ASSOCIATED FCRL4\(^+\) B CELLS IN PRIMARY SJÖGREN’S SYNDROME REVEALS A PATHOGENIC SIGNATURE

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Work in progress
ABSTRACT

Objective
A small subset of B cells in blood expresses Fc-receptor-like protein 4 (FcRL4). In healthy individuals, FcRL4 expression is mostly limited to B cells in mucosal tissues. In patients with primary Sjögren's syndrome (pSS), FcRL4+ B cells are found in inflamed salivary gland tissue, in particular within parotid glands. At these sites, FcRL4+ cells reside in close proximity to ductal epithelial cells. FcRL4 is also expressed by mucosa-associated lymphoid tissue (MALT) lymphoma B cells. We aimed to assess the phenotype and frequency of circulating FcRL4+ B cells as well as the transcriptional profile of FcRL4+ B cells from parotid glands of pSS patients.

Methods
The frequency and phenotype of FcRL4+ B cells in blood was assessed by flow cytometry. Peripheral blood mononuclear cells of 44 pSS patients and 54 non-SS sicca patients were included. Additionally, fresh parotid gland biopsies from 6 pSS patients were obtained. Of these biopsies cell suspensions were prepared, which were incubated with appropriate antibodies and sorted as single-cells or 5 cells per well ('bulk') based on the following definitions: CD19+CD27-FcRL4- ('naive') CD19+CD27+FcRL4- ('memory') and CD19+FcRL4+. Preparation of cDNA libraries was done using an in-house SMARTseq2 protocol and sequencing was performed on an Illumina NextSeq500.

Results
Frequencies of circulating FcRL4+ B cells were not significantly altered in pSS patients compared with non-SS sicca controls. The majority of FcRL4+ B cells in pSS patients and controls had a CD21loCXCR3+ phenotype. For RNA sequencing analysis of glandular FcRL4+ B cells, samples from 5 out of 6 pSS patients passed quality control. Of these 5 patients, one was diagnosed with MALT lymphoma and therefore excluded from differential expression analysis. From the remaining patient samples a total of 150 single B cells and 360 B cells in ‘bulk’ were included in the analysis. Both in single-cell and ‘bulk’ analysis, multiple genes coding for integrins, such as ITGAX (CD11c), were significantly upregulated in FcRL4+ B cells. Gene Ontology pathways that showed the highest upregulation in FcRL4+ B cells (both single-cell and ‘bulk’) were receptor binding, GTPase and protein kinase pathways. In ‘bulk’ samples genes encoding for Src tyrosine kinases, genes involved in the NF-κB pathway, CXCR3, and TNFRSF13B (TACI) were significantly upregulated in FcRL4+ B cells, compared with either CD27 FcRL4+ or CD27+FcRL4+ B cells. Gene expression levels of CD40 and LCK were significantly decreased in FcRL4+ B cells.
Conclusion
Circulating FcRL4+ B cells are mostly activated cells, reflected by a CD21loCXCR3+ phenotype. The frequency of these cells was similar in pSS patients and non-SS sicca controls. FcRL4+ B cells in salivary glands of pSS patients show upregulation of genes involved in homing and cell adhesion, consistent with their location close to the epithelium. FcRL4+ B cells also show upregulation of genes that promote inflammation and B cell survival, possibly in a T cell-independent manner. These cells exhibit all characteristics of chronically activated CD11c+ memory B cells. We postulate that these cells contribute significantly to the epithelial damage seen in the glandular tissue of pSS patients.
INTRODUCTION

Primary Sjögren’s syndrome (pSS) is a systemic autoimmune disease that affects about 0.04% of the general population [1], and predominantly woman. Salivary and lacrimal glands are the main target of the disease. Patients usually present with symptoms of dry mouth, dry eyes, and fatigue. Mononuclear infiltration, mainly consisting of CD4+ T cells and B cells, is a characteristic histopathological finding in pSS patients. Infiltrates are mostly found around the ductal epithelium, but lymphocytes can even be present within the epithelium where they may become part of lymphoepithelial lesions (LEL) [2].

LELs are composed of proliferative metaplastic epithelial cells and intraepithelial lymphocytes, mostly B cells. The vast majority of these intraepithelial B cells express the inhibitory IgA-binding Fc receptor-like protein 4 (FcRL4) [3,4]. In healthy individuals, FcRL4+ B cells are restricted to mucosal tissues and mesenteric lymph nodes, where they participate in mucosal immune responses [5]. Interestingly, FcRL4+ B cells have also been found in the synovium of patients with rheumatoid arthritis (RA), and here they produce high levels of RANKL, thereby exhibiting a pathogenic role in this disease [6,7]. Analysis of immunoglobulin genes from FcRL4+ B cells in RA showed high levels of hypermutation in the variable regions [6], which indicates involvement of antigen and T cells in the selection of these cells. Synovial FcRL4+ B cells from RA patients harbored autoreactive clones and mostly used the IgG1 and IgA1 isotypes. Usage of the IgA isotype was more frequent in FcRL4+ compared with FcRL4− B cells [6]. These studies in RA patients indicate that FcRL4+ B cells are activated cells that may play a major role in local immune responses in autoimmune diseases, and that these cells can contribute to tissue damage.

Patients with pSS have a significantly increased risk for development of non-Hodgkin lymphomas, mostly of the mucosa-associated lymphoid tissue (MALT) type [8]. MALT lymphomas in pSS develop preferentially in the parotid glands. The neoplastic B cells of parotid MALT lymphomas are located in and around LELs, and widely express FcRL4 [9]. Hence, we postulated that intraepithelial FcRL4-expressing B cells may be the cells from which MALT lymphomas in parotid glands arise.

Whether glandular FcRL4+ B cells have been activated at mucosal tissue sites (e.g., gut mucosa) and subsequently migrate to the inflamed salivary glands of pSS patients, or whether they are formed locally in these glands is not known. Also their functional properties within the inflamed salivary glands are not clear. For these reasons we studied the phenotype and frequency of circulating FcRL4+ B cells in pSS patients by flow cytometry, and the transcriptome of local FcRL4+ B cells by single cell RNA sequencing, to gain insight in the phenotype and functional capabilities of these cells. We hypothesized that FcRL4+ B cells migrate into the inflamed glands of pSS patients.
and contribute to pathogenesis by participating in LEL formation and by secretion of pro-inflammatory factors. Furthermore, we hypothesized that FcRL4+ B cells express genes that may predispose these cells to lymphomagenesis.

PATIENTS AND METHODS

Immunophenotyping of circulating B cells

Consecutive patients, referred to the Sjögren Expertise Center of the University Medical Center Groningen (UMCG) for suspicion of SS were included (n=98). Informed consent was obtained from all patients according to the Declaration of Helsinki and the study was approved by the Medical Research Ethics Committee of the UMCG (METc2013.066). Patients that fulfilled 2016 ACR-EULAR criteria for pSS were classified as pSS patients [10]. From the 98 patients included in our cohort, 44 patients were classified as pSS and 54 as non-SS sicca patients. All pSS patients were naive for treatment with biologic disease-modifying anti-rheumatic drugs. Two pSS patients were diagnosed with MALT lymphoma. Cryopreserved peripheral blood mononuclear cells were thawed and analyzed by flow cytometry for the presence of circulating FcRL4+ B cells. The following antibodies were used: anti-human-CD19-BV786 (clone SJ25C1), anti-human-CD27-BV421 (clone M-T271), anti-human-IgD-BUV395 (clone IA6-2), anti-human-CD21-BUV737 (clone B-Iy4), anti-human-CXCR3-PE-Cy7 (clone 1C6), all from BD Biosciences, and anti-human-FcRL4-PE (clone 413D12, Biolegend). Fixable viability dye eF506 (eBioscience) was used for live/dead discrimination. Data were acquired on a FACS-LSRII flow cytometer (Becton Dickinson, USA) and analyzed using FlowJo software (Tree Star, USA).

Tissue samples for RNA sequencing

Parotid gland tissue was obtained fresh from 6 adult patients who were anti-SSA positive, had a high clinical suspicion of pSS, and underwent a diagnostic biopsy. All surgeries were performed at the department of Oral and Maxillofacial Surgery of the University Medical Center Groningen (UMCG), the Netherlands. Permission to collect these tissues for research purposes was obtained from the Medical Research Ethics Committee of the UMCG (METc2016/010).

Preparation of cell suspensions for RNA sequencing

Cell suspensions were prepared as described by Pringle et al. [11], with the following adaptations: Biopsies were manually cut using scissors, and the incubation period for enzyme-based digestion was 30 minutes. 32.5 μL digestion buffer was used per milligram of tissue. Cells were resuspended in MACS buffer (PBS/0.5% BSA/2 mM EDTA) for cell surface staining.
Flow cytometry analysis and sorting for RNA sequencing

Cell suspensions prepared from parotid gland tissue were incubated with antibodies (identified below) for 30 min at 4 °C, and washed twice in MACS buffer. The following antibodies were used: anti-human-CD19-eF450 (clone HIB19), anti-human-CD27-APC (clone O323), both from eBioscience, and anti-human-FcRL4-PE (clone 413D12, Biolegend). Immediately before sorting, cells were stained with Propidium Iodide (eBioscience) for live/dead discrimination. Gating was performed as described in supplementary figure 1. Cells were sorted by 1 or 5 cells/well into 96-well PCR plates containing 2 μl of lysis buffer (0.2% Triton X-100 (Sigma-Aldrich) + 2 U/μL RNase inhibitor (Westburg-Clontech)), 1 μl of 10 μM oligo-dT_{30}VN primer (Biolegio) and 1 μl of 4 x 10mM dNTP mix (Westburg-Fermentas) per well. Cells were sorted on a MoFlo Astrios cell sorter (Beckman Coulter).

Preparation of cDNA libraries and sequencing

For cDNA library preparation the Smart-seq2 protocol by Picelli et al. was used [12,13], with the following adaptions: Reverse Transcriptase (RT) mastermix contained: 2.5 U SmartScribe RT, 0.25 U RNAse inhibitor (both from Westburg-Clontech), 1x SmartScribe first-strand buffer, 2 nM DTT (both from LifeTechnologies), 1 M Betaine (BioUltra ≥99.0%; Sigma-Aldrich), 1μM BC-TSO (Biolegio). After reverse transcriptase an exonuclease step was added to remove unbound oligo-dT primers. One μL of Exonuclease I (1:400 dilution in pure water) was added to each well and the plate was incubated 45 minutes at 37 °C, to activate the enzyme, immediately followed by 15 minutes at 85 °C to inactivate the enzyme. Samples were purified using Agencourt Ampure XP Beads (Beckman Coulter). The presence and size distribution of the obtained PCR product was measured on a PerkinElmer LabChip GX high-sensitivity DNA chip. Next, PCR products were pooled for tagmentation. Products from single cells were pooled 1:1, and equimolar pooling was performed for products from 5 cells/well. To tag the DNA with adapter sequences, a tagmentation step was performed using the Illumina Nextera XT DNA sample preparation kit, according to the manufacturer’s protocol, with 500 pg of pooled cDNA. Subsequently, the subpools were indexed using a N7xx primer from the Nextera XT DNA sample preparation kit and a custom P5-TSO hybrid primer (10 uM) with the Nextera PCR mastermix. The concentration and size distribution of the obtained Nextera products were measured on a PerkinElmer LabChip GX high-sensitivity DNA chip. Next, a superpool was prepared by equimolar pooling of the six Nextera products. The superpool was divided over four lanes and sequenced on a Illumina NextSeq500 instrument. The first read consisted of 18 bp, to sequence the cell-barcode (10 bp) and the UMI (7 bp), followed by the index read sequencing the Nextera index (8 bp). The second read consisted of the Nextera index plus 50 bp, sequencing the last part of the
captured gene. For single cells, the average sequencing depth was 4.2x10^6 reads per cell.

**Read alignment, quality control and gene expression estimation**

Dropseq tools 1.12 was used to extract well barcodes and molecular barcodes (unique molecular identifiers; UMI's) from the reads [14]. The extracted well and UMI reads are flagged with BAM tags and then stored in BAM format together with the corresponding read. For quality control only reads with a well and UMI barcode with a minimum basecallQuality of 10 were included. Other reads were discarded. Of the remaining reads the SMART adapter and PolyA tails were removed using Dropseq. In the next step, reads were aligned to the human genome (hg19) using STAR v2.5.1b with default settings [15]. The aligned reads were filtered for uniquely mapping reads. Before gene quantification, aligned reads were sorted using Picard tools 2.2.2 (https://broadinstitute.github.io/picard). Ensembl version 75 was used to map protein-coding transcripts. For single cells, quantification of gene expression was performed using Dropseq filtering on unique UMI's.

**Data analysis**

All statistical analysis and plotting of RNA-seq data was performed using R software. For single cells, reads with unique molecular identifiers (UMI) were used as input, to correct for PCR duplicates [16]. Seurat version 2.1 was used for quality control, clustering and analysis of single cell data [17]. Single cells that expressed <200 genes and genes that were expressed in <3 cells overall were excluded. Also cells with a mitochondrial gene percentage higher than three times the median absolute deviation across all cells were excluded [18]. For differential expression analysis of single cell data, the MAST package was used [19]. For differential expression analysis of ‘bulk’ (5 cells/well) samples, DESeq2 was used [20], with 6 biological replicates per sample. Raw read counts (not corrected for UMI duplicates) were used as input, because DESeq2 expects un-normalized counts, and internally corrects for library size. P-values were adjusted by the Benjamini-Hochberg method and are hereafter indicated as false detection rate (FDR).

**RESULTS**

1. **Frequency and phenotype of circulating FcRL4⁺ B cells in pSS patients and non-SS sicca patients**

First, we compared the frequencies of circulating FcRL4⁺ B cells between pSS patients and non-SS sicca patients. Additionally, we measured co-expression of multiple B cell-related markers, including CD27, CD21, and CXCR3 (Figure 1A). We found no significant
difference in frequencies of circulating FcRL4+ B cells between pSS patients and non-SS sicca patients (Figure 1B). Also absolute numbers of these cell subsets were not significantly altered (data not shown). Two pSS patients with MALT lymphoma did not show aberrant frequencies of these cells either. Circulating FcRL4+ B cells comprised both CD27- ‘naive’ and CD27+ ‘memory’ cells. A large proportion of circulating FcRL4+ B cells expressed low levels of CD21 and co-expressed CXCR3 (Figure 1B). We did not observe significant phenotypical differences in FcRL4+ B cells between pSS patients and non-SS sicca patients. Albeit their low prevalence, the phenotype of circulating FcRL4+ B cells suggests that these cells have the capacity to migrate to inflamed tissue sites by expression of CXCR3.

**FIGURE 1 | FcRL4+ B cells in peripheral blood of pSS patients and non-SS sicca patients.** (A) Gating strategy used to identify FcRL4+ B cells in peripheral blood. Lymphocytes were gated from single, live cells using forward and side scatter properties and fixable viability dye staining. (B) Frequencies of FcRL4+ cells within the B cell compartment and frequencies of CD21loCXCR3+ cells within the FcRL4+ B cell compartment are shown. Data from pSS patients (n=44) and non-SS sicca patients (n=54) were included. P-value<0.05 was considered significant. Mann-Whitney U test was used for statistical analysis. Ns = not significant.

**2. RNA sequencing of B cells isolated from salivary glands of pSS patients**

To elucidate the phenotype and function of local FcRL4+ B cells, we sorted these cells, as well as FcRL4- B cell subsets, from parotid gland tissues and isolated RNA for sequencing. Gene expression profiles were compared between FcRL4+, FcRL4 CD27- (‘naive’) and
FcRL4 CD27+ (‘memory’) B cells. Expression data of ‘bulk’ samples (5 cells/well) and single cells were analyzed separately. One patient (out of 6) was excluded before sequencing because of low RNA yields during library preparation for the ‘bulk’ samples. Single cells from this patient that were sequenced clustered highly distinct from other patients and were also excluded. Of the remaining 5 patients, one was diagnosed with MALT lymphoma. Data from this patient were excluded from differential expression analysis, because the transcriptional profile of these cells may bias the analysis of non-lymphoma B cells.

### 2.1 Differential expression analysis of ‘bulk’ cells.

Bulk cells (5 cells per subset per patient, 6 replicates) from all 4 patients were pooled in order to compare the gene expression profiles between subsets. In total 360 cells were included in the analysis. Gene Ontology pathway analysis using the PANTHER classification system[21,22] showed differential expression in receptor binding, ATPase, GTPase and protein kinase pathways in FcRL4+ B cells, compared with FcRL4-CD27- and FcRL4-CD27+ B cells. By using DESeq2 software to compare FcRL4+ B cells with FcRL4-CD27- (‘naive’) and FcRL4-CD27+ (‘memory’) B cells, we identified 1067 and 620 differentially expressed genes (FDR<0.05), respectively. Volcano plots in figures 2A and 2B illustrate the top differentially expressed genes based on significance level. The most distinctly expressed genes in FcRL4+ B cells, compared with FcRL4-CD27- or FcRL4-CD27+ B cells, were FCRL4 (up) and RGS16 (down).

Next, we evaluated significantly differentially expressed genes with known immune function (Figure 2C). We found differential expression of several transcripts previously associated with FcRL4+ B cells [6,23]. These transcripts include the integrin ITGAX (CD11c), Src tyrosine kinases HCK and FGR (all up), LCK and CXCR5 (down). We also found upregulation of multiple genes associated with activation of the NF-kappa B (NF-kB) signaling pathway: NFKB1 (p50), BCL2A1, MAP3K14 (NIK) and TRAF3, indicating that both canonical and non-canonical NF-kB pathways are active. Expression levels of NFKBIA (IκBα) and NFKBID (IκBNS), negative regulators of NF-kB, were significantly downregulated in FcRL4+ B cells. Other genes upregulated in FcRL4+ B cells were THEMIS2, IL27RA, CD97, CXCR3, JAK2, and TNFRSF13B (TACI). Downregulated genes included MZB1 and CD40. Several genes with known immune function were only differentially expressed when comparing FcRL4+ B cells with FcRL4-CD27- (‘naive’) B cells. These genes include: TGFB1 , IL18BP, IRF2, POLB, CD86, MYD88, TNF and SYK (all up). These genes are apparently actively transcribed by both FcRL4+ B cells and FcRL4-CD27+ ‘memory’ B cells. A list of all differentially expressed genes with FDR<0.05 is available upon request from the corresponding author.
FIGURE 2 | Differential gene expression in FcRL4+ B cells at ‘bulk’ level. Differential gene expression for 5 cells/well samples was calculated using DESeq2 software. Volcano plots illustrate significantly differentially expressed genes (FDR<0.05, red) against non-significant genes (black). Differentially expressed genes in FcRL4+ B cells versus FcRL4-CD27- B cells (A), or versus FcRL4-CD27+ B cells (B) are displayed. In each volcano plot, ten genes with the lowest false detection rate (FDR) value are labeled. For each gene, the negative log10-transformed p-value is plotted on the y-axis and the log2-transformed fold change is plotted on the x-axis. A positive fold change indicates upregulated expression in FcRL4+ B cells. (C) Violin plots showing normalized counts (y-axis) per cell type (x-axis) for genes with known immune function that were significantly differentially expressed. Each black dot represents a 5-cell sample.
2.2 Differential expression analysis of single cells

Out of 159 sequenced parotid gland B cells, 9 cells were excluded after quality control using the Seurat package, mainly due to a high mitochondrial gene percentage. Thus, from 4 patients 150 single cells in total were included in the analysis. The lowest number of cells per B cell subset per patient was six, the highest 16. To remove unwanted sources of variation in gene expression, data were scaled by regressing out the number of unique gene transcripts (the number of UMIs) per cell and the percentage of mitochondrial RNA per cell. Subsequently, unbiased clustering analysis was performed to assess if flow cytometry-defined cell subsets showed different gene expression patterns. When the first 12 principal components were projected onto two-dimensions by t-distributed stochastic neighbor embedding (t-SNE) [24], almost all flow cytometry-defined cell subsets per patient clustered separately (supplementary figure 2). In one patient, FcRL4+ cells and FcRL4-CD27+ cells did not separate well based on tSNE cluster 1 and 2, which may indicate that in this patient the gene expression profile between FcRL4+ cells and FcRL4-CD27+ cells is not highly distinct.

For differential expression analysis, we combined the various flow cytometry-defined cell subsets of all 4 patients. By using MAST software to compare FcRL4+ B cells with FcRL4-CD27- (‘naive’) and FcRL4-CD27+ (‘memory’) B cells, we identified 272 and 205 differentially expressed genes (FDR<0.05), respectively. Almost similar to the ‘bulk’ samples, Gene Ontology pathway analysis of single cells showed differential expression in receptor binding, nucleic acid binding, GTPase and protein kinase pathways in FcRL4+ B cells, compared with FcRL4-CD27- and FcRL4-CD27+ B cells. Volcano plots in figure 3A and 3B illustrate significantly differentially expressed genes against non-significant genes. **FCRL4, ITGAX, ITGB7, MLF2** and **TNFRSF13B** (TACI) were genes with known immune function that were significantly upregulated in FcRL4+ B cells, compared with both FcRL4- subsets (Figure 3C). The following genes were differentially expressed in FcRL4+, compared with FcRL4-CD27- B cells: **ISG15, IRF7, OAS2, CD27, AICDA, SLAMF1** and **TGFB2** (all upregulated). **BCLAF1** (transcriptional repressor of BCL2 family of proteins) and **PIK3IP1** (negative regulator of PI3K activity) were significantly downregulated. When comparing FcRL4+ with FcRL4-CD27+ B cells, **CD84, IFI44, BCL2A1, PLCG2, CIITA, FGR, TNFSF10, TLR1**, and **CD48** were significantly upregulated. Expression levels of **IRF7** and **AICDA** were also upregulated, but not significantly (FDR=0.07 and 0.18, respectively). **CXCR4, CCR7** and **POLD2** were significantly downregulated. There were no genes with known immune function that were significantly downregulated in FcRL4+ cells compared to both FcRL4- subsets. Although overlap in results is seen, several genes that were differentially expressed in the ‘bulk’ samples (figure 2C), were only rarely detected in the single cell samples.
DISCUSSION

Recently, we showed that FcRL4 is expressed by intraepithelial B cells in the salivary glands of pSS patients. The origin, phenotype and functional capabilities of FcRL4+ B cells in the inflamed glandular tissue remain, however, poorly understood. Given their potential role in pSS pathogenesis, we assessed the frequency and phenotype of FcRL4+ B cells...
B cells in the circulation of pSS patients, and investigated the transcriptional profile of FcRL4+ B cells located in the inflamed parotid glands. We observed that frequencies of circulating FcRL4+ B cells were generally low, and no difference in number or proportion of these cells was found between pSS patients and non-SS sicca patients. Albeit their low prevalence, we found that a large proportion of circulating FcRL4+ B cells expressed low levels of CD21 and co-expressed CXCR3, indicating that these cells are activated and programmed to migrate to inflamed tissues. In addition to immunophenotyping of circulating FcRL4+ B cells, we investigated, for the first time, the gene expression profile of glandular FcRL4+ B cells, isolated from parotid gland tissue of 4 pSS patients. We found multiple upregulated pathways that are involved in cell signaling, including receptor binding, GTPase and protein kinase pathways. Differentially expressed genes with known immune function could be subdivided into homing, B cell activation and lymphomagenesis pathways.

Homing
Gene expression analysis of the ‘bulk’ (5 cells) samples showed upregulation of CXCR3 in FcRL4+ B cells, together with downregulation of CXCR5. Similarly, flow cytometric analysis of circulating B cells showed that a large proportion of FcLR4+ B cells in blood co-express CXCR3. This may explain homing to the ductal epithelial cells of the salivary glands, which secrete high levels of the chemokine CXCL10/IP-10, the ligand for CXCR3. Additionally, integrins (e.g., ITGAX (CD11c)) and adhesion molecules (e.g., CD97) were upregulated in FcRL4+ B cells, compared with FcRL4- B cells. The expression of integrins and adhesion molecules by FcRL4+ B cells may result in retention of these cells around and within the epithelium by interaction with their ligands, such as ICAM-1. FcRL4+ B cells further exhibited increased transcript expression of Src family kinases (HCK, FGR, LYN), which are important for integrin signal transduction [25]. The upregulated expression of ITGAX (CD11c) and Src family kinases in glandular FcRL4+ B cells is consistent with results from earlier studies that analyzed the transcription profile of tonsillar FcRL4+ B cells or FcRL4+ B cells from synovia of patients with RA [6,23]. Upregulation of Src family kinases can contribute to antibody-induced inflammation, as mice that lack Src tyrosine kinases are protected from autoantibody-induced arthritis [26].

B cell activation
Previous studies have shown that enhanced expression of CD11c by memory B cells is associated with multiple autoimmune conditions and chronic immune stimulation [27]. CD11c+ memory cells are atypical memory cells, characterized by low expression of CD27 and CD21 [27]. These cells contain autoreactive specificities, are refractory to BCR stimulation, and respond robustly to TLR activation [27]. A similar pattern of
downregulated BCR signaling and enhanced TLR signaling is seen in FcRL4+ B cells [28]. There is some evidence that binding of IgA to FcRL4 on the B cells is important for this switch from adaptive to innate signaling [28]. Negative regulation of BCR-induced signaling may be established by upregulation of LYN, as we observed in these cells. Lyn is a Src tyrosine kinase that can initiate, but also negatively regulate BCR signaling [29]. The transcriptional profile of FcRL4+ B cells from the parotid glands further indicates that these cells have indeed been activated, possibly via TLR stimulation, as upregulation of multiple genes involved in both canonical and non-canonical NF-κB signaling was seen in the ‘bulk’ samples. Upregulation of genes involved in NF-κB signaling seems specific for FcRL4+ B cells residing in the inflamed glandular tissue of pSS patients, since this was not reported for tonsillar FcRL4+ B cells or synovial FcRL4+ B cells [6,23]. Unexpectedly, the upregulation of NF-κB pathway genes in glandular FcRL4+ B cells was not accompanied by increased expression levels of genes encoding for pro-inflammatory cytokines such as TNFα or IL-6. FcRL4+ B cells did express higher levels of TNF compared to FcRL4-negative naive B cells, but not compared to FcRL4-negative memory B cells. Thus, effector functions of FcRL4+ B cells that may contribute to epithelial damage and formation of LELs remain unclear. Another gene that was upregulated in FcRL4+ B cells and that is involved in B cell activation and survival is TACI. Binding of BAFF/Blys or APRIL to TACI can enhance NF-κB signaling and promote B cell survival [30,31]. These cytokines are significantly upregulated in the salivary glands of pSS patients and are also produced by the ductal epithelial cells [32]. Binding of BAFF and/or APRIL to TACI expressed by FcRL4+ B cells may promote their activation and survival. A less well-known gene involved in B cell activation that was also significantly upregulated in FcRL4+ B cells was THEMIS2. Recently it was shown that THEMIS2 reduces the threshold for B cell activation by low-avidity antigens such as soluble proteins [33], which is clearly unfavorable under autoimmune conditions. Interestingly, this study also showed that Themis2 interacts with Lyn, which was also upregulated in FcRL4+ B cells. Altered expression of THEMIS2 indicates that BCR signaling is modulated in FcRL4+ B cells, although the implications for B cell activation of these cells remain to be elucidated.

**Lymphomagenesis**

FcRL4 is not only expressed by epithelium-associated B cells, but also by MALT lymphomas [9]. We therefore speculated that the highly proliferative [3], activated FcRL4+ B cells may become neoplastic B cells. In support of this notion, we observed upregulation of several genes in FcRL4+ B cells that are associated with lymphomagenesis. Firstly, expression levels of several NF-κB pathway genes were increased and all MALT lymphoma-associated gene translocations are associated with NF-κB activation [34]. An additional factor that may contribute to lymphomagenesis is upregulation of genes that promote cell survival (TACI, BCL2A1, MLF2) in FcRL4+ B cells,
probably saving them from apoptosis. Furthermore, the most strongly downregulated gene in FcRL4+ B cells in the ‘bulk’ samples was RGS16. This gene was not differentially expressed in tonsillar or synovial FcRL4+ B cells [6,23]. RGS16 belongs to the family of regulators of G protein signaling (RGS), acting as GTPase activating proteins. RGS16 is involved in negative regulation of several oncogene pathways, including EGF/EGFR, MAPK, AKT/PI3K, RhoA, and SDF-1/CXCR4 in normal or cancer cell lines [35]. In addition, RGS16 was downregulated in mantle cell lymphoma B cells compared with naïve B cells [36], suggesting that the absence of RGS16 in FcRL4+ B cells may play a role in lymphomagenesis. The physiological role of RGS16 is, however, poorly understood and needs further investigation. A different gene that was strongly upregulated in FcRL4+ B cells is IL27RA. The expression level of this gene was previously shown to be increased in MALT lymphoma compared with other B cell lymphomas [37]. In mice, it was shown that IL-27 signals directly to B cells and promotes differentiation towards germinal center (GC) B cells via STAT1 [38]. In line with these findings, IL-27R was induced on GC B cells following CD40 stimulation and induced STAT1 phosphorylation in humans [39]. IL-27 could also induce T-bet expression in naïve and memory B cells [39,40]. Of interest, in the single cell samples, we identified a small amount of FcRL4+ B cells that expressed AICDA. Consistent with these findings, a previous study showed that FcRL4+ memory B cells have higher expression levels of AICDA than FcRL4− memory B cells [23]. The AICDA gene is coding for activation-induced cytidine (AID), which is essential for somatic hypermutation and isotype switching in GC B cells. Recently it was shown that AID can also target many other genes outside immunoglobulin loci, which may result in off-target, potentially oncogenic, mutations [41]. Prolonged expression of AICDA in FcRL4+ B cells may therefore contribute to transformation of FcRL4+ B cells towards neoplastic MALT lymphoma cells. The clear co-localization of neoplastic B cells with epithelial cells in MALT lymphomas suggests that this disease depends on the interaction between B cells and epithelial cells.

A limitation of our study is the small amount of single cells that were sorted and sequenced. Numbers of FcRL4+ cells within the infiltrate are relatively low, and for ethical reasons only a small-sized biopsy could be obtained for research purposes. Due to the stochasticity of gene expression, in combination with a high proliferation rate of FcRL4+ B cells [3], our differential expression analysis in single cells may be underpowered. Nonetheless, to our best knowledge this is the first study to reveal the gene expression profile of FcRL4+ B cells isolated from salivary gland tissue of pSS patients. We show that these cells exhibit all characteristics of chronically activated CD11c+ memory B cells, including integrin expression, which may be responsible for cross-talk with epithelial cells that form LELs. By interacting with epithelial cells, FcRL4+ B cells may contribute to pSS histopathology and hyposalivation. Lastly, we show that FcRL4+ B cells isolated from glandular tissue of pSS patients express anti-apoptotic factors that, combined with
ongoing somatic hypermutation and a high proliferative capacity, may put them at risk of lymphomagenesis.

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SUPPLEMENTARY FIGURE 1 | Gating strategy for sorting of B cell subsets from parotid gland tissue. (A) Lymphocytes were selected based on forward and side scatter properties. (B) Dead cells were excluded based on propidium iodide (PI) staining. (C) B cells were selected based on positive staining for CD19. (D) Three B cell subsets were sorted: CD27 FcRL4-, CD27+/FcRL4-, and CD27+/FcRL4+ cells.
SUPPLEMENTARY FIGURE 2 | T-distributed stochastic neighbor embedding (tSNE) plot of all single cell samples after unbiased clustering. Cells are colored by flow-cytometry based cell subsets.