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Transcription profiling of peripheral B cells in antibody-positive primary Sjögren's syndrome reveals upregulated expression of *CX3CR1* and a type I and type II interferon signature

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Abstract

B cells play a key role in the pathogenesis of primary Sjögren's syndrome (pSS). The aim of this study was to analyse the transcriptome of CD19+ B cells from patients with pSS and healthy controls to decipher the B cell–specific contribution to pSS. RNA from purified CD19+ B cells from 12 anti-SSA antibody-positive untreated female patients with pSS and 20 healthy blood donors was subjected to whole transcriptome sequencing. A false discovery rate corrected significance threshold of $\alpha < 0.05$ was applied to define differential gene expression. As validation, gene expression in B cells from 17 patients with pSS and 16 healthy controls was analysed using a targeted gene panel. RNA-sequencing identified 4047 differentially expressed autosomal genes in pSS B cells. Upregulated expression of type I and type II interferon (IFN)-induced genes was observed, establishing an IFN signature in pSS B cells. Among the top upregulated and validated genes were *CX3CR1,* encoding the fractalkine receptor involved in regulation of B-cell malignancies, *CCL5*/RANTES and *CCR1*. Increased expression of several members of the TNF superfamily was also identified; *TNFSF4*/Ox40L, *TNFSF10*/TRAIL, *TNFSF13B*/BAFF, *TNFRSF17*/BCMA as well as *S100A8* and *−A9*/calprotectin, *TLR7*, *STAT1* and *STAT2*. Among genes with downregulated expression in pSS B cells were *SOCS1* and *SOCS3*, *CD70* and *TNFAIP3*/A20. We conclude that B cells from patients with anti-SSA antibody-positive pSS display immune activation with upregulated expression of chemokines, chemokine receptors and a prominent type I and type II IFN signature, while suppressors of cytokine signalling are downregulated. This adds insight into the autoimmune process and suggests potential targets for future functional studies.

1 | INTRODUCTION

B cells play a central role in the pathogenesis of primary Sjögren's syndrome (pSS), a systemic autoimmune disease characterized by inflammation of salivary and lacrimal glands. B cell activity in pSS is reflected by hypergammaglobulinemia and the presence of autoantibodies in sera against the ribonucleoproteins SSA/Ro and SSB/La.[1] Histopathological examination of minor salivary glands identifies focal lymphocytic infiltrates, which show germinal centre-like structures with B cell maturation and local anti-SSA/SSB antibody synthesis in a third of the patients.[2] The presence of germinal centre-like formations in the minor salivary gland infiltrates has also been proposed as a possible predictor for lymphomagenesis.[3] Patients with pSS have a 16-fold increased risk of developing lymphoproliferative malignancies, most commonly non-Hodgkin lymphomas of the B cell type.[4]

The aetiology of pSS is considered to be multifactorial, where environmental, genetic and epigenetic factors contribute to disease development. We have previously detected hypomethylation at interferon (IFN)-regulated genes in multiple tissues from patients with pSS, which corresponded to an increased mRNA expression of the most hypomethylated genes in CD19+ B cells from patients with pSS.[5] Upregulation of IFN-induced genes, a so-called IFN signature, has also been observed by others in whole blood, different peripheral blood mononuclear cell types and salivary gland tissue from patients with pSS using reverse transcriptase quantitative PCR (RT-qPCR) and gene expression microarrays.[6-11] Gene expression profiling of naïve B cells from untreated patients with pSS revealed an IFN signature that was largely abolished in B cells from hydroxychloroquine-treated patients with pSS.[11] Analysis of gene expression in minor salivary glands from patients and controls, and patients before and after treatment with rituximab, reported differentially expressed genes in IFN- and B cell signalling pathways.[6,10,12] However, as lymphocyte infiltration of exocrine glands is a hallmark of pSS, expression profiles derived from salivary gland tissue may eventually reflect differences in cell type composition rather than differential gene expression. Given the multiple roles of B cells in pSS, the aim of this study was to apply RNA-sequencing in a comprehensive analysis of the whole transcriptome of primary CD19+ B cells from patients with pSS to increase the understanding of the regulatory molecular pathways in pSS pathogenesis. The advantage of RNA-sequencing compared with expression arrays is the more sensitive and unbiased detection of all expressed transcripts.

2 | MATERIALS AND METHODS

2.1 | Patients and controls

For RNA-sequencing of CD19+ B cells, a total of 12 Caucasian female patients with pSS (mean age 61.2 ± 9.1 years) from the Rheumatology Clinic at Uppsala University Hospital, Sweden, all fulfilling the American European Consensus Group (AECG) criteria, were included.[13] Clinical data were obtained from medical records. All patients were positive for anti-SSA antibodies in their sera, none had a previous lymphoma, and all were untreated regarding immunomodulatory drugs including prednisolone, hydroxychloroquine, immunosuppressants and biologics (Table 1). Disease activity was assessed using the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI), and a low disease activity was defined as ESSDAI < 5.[14] As controls, 20 B cell samples were obtained from age- and ethnicity-matched healthy blood donors (mean age 57.8 ± 12.1 years) from the Uppsala Bioresource.[15] The validation cohort consisted of 17 untreated anti-SSA antibody-positive female patients with pSS, all fulfilling AECG criteria, from the Rheumatology Clinic at the Karolinska University Hospital, Stockholm, Sweden and 16 age- and ethnicity-matched healthy laboratory personnel controls (Table S1). The study was approved by the Regional Ethics Board in Uppsala No. 97358, 217/2006 and 013/2009, and the Regional Ethics Committee Stockholm North No. 2008/915-31/4 and 2015/1811-32/4. All patients and controls gave written informed consent to participate, and the study was carried out in compliance with the principles expressed in the Declaration of Helsinki.

2.2 | Sample preparation

For RNA-sequencing, CD19+ B cells were fractionated from buffy coats applying Ficoll-Hypaque density-gradient centrifugation (GE Healthcare, Uppsala, Sweden) for PBMC isolation followed by positive selection with CD19-specific microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the isolated cell population (>95%) was confirmed by control sampling via flow cytometry (FACSCanto II, BD Biosciences, Stockholm, Sweden). For DNA and RNA preparation, the AllPrep DNA/RNA Mini Kit and the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) were used. In the validation cohort, CD19+ B cells were isolated from PBMCs obtained using Vacutainer CPT Mononuclear Cell Preparation Tubes (BD Biosciences) in an autoMACS Pro Separator (Miltenyi Biotec) utilizing positive selection

TABLE 1 Clinical characteristics of anti-SSA–positive female patients with pSS with CD19+ B cells analysed by RNA-sequencing

Patients with pSS	Age at diagnosis	Age at B-cell sampling	Antibodies	Focus score[a]	ESR mm/h	P-IgG g/L	Cumulative extraglandular manifestations[b]	ESSDAI at B-cell sampling	ESSDAI domains
pSS 1	67	70	ANA, SSA, SSB	5	72	46.3	Leucopenia, Raynaud's, CNS	2	Biological
pSS 2	61	64	ANA, SSA	1	22	12.0	None	0	
pSS 3	64	66	ANA, SSA	1	36	12.2	Salivary gland swelling	0	
pSS 4	61	62	SSA	5	36	31.2	Leucopenia, hypothyroidism, arthralgia	4	Articular
pSS 5	43	44	ANA, SSA	7	17	15.8	Leucopenia	0	
pSS 6	64	65	SSA	n.a.	27	n.a.	None	0	
pSS 7	54	55	SSA	1	8	9.0	Leucopenia, Raynaud's, salivary gland swelling	2	Haematological
pSS 8	41	48	ANA, SSA, SSB	2	11	12.6	Leucopenia, lymphadenopathy	4	Lymphadenopathy
pSS 9	50	66	ANA, SSA, SSB	8	14	n.a.	none	0	
pSS 10	65	76	ANA, SSA, SSB	2	53	15.1	Leucopenia, Raynaud's, cutaneous vasculitis	11	Cutaneous, haematological, biological
pSS 11	42	55	ANA, SSA, SSB	3	10	n.a.	None	0	
pSS 12	54	64	ANA, SSA	2	6	15.9	Leucopenia, salivary gland swelling, hypothyroidism	0	

ANA, antinuclear antibodies; ESR, erythrocyte sedimentation rate, ESSDAI, European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index; n.a., not analysed.

[a]Minor salivary gland focus score defined as number of foci with >50 lymphocytes/4 mm^2.

[b]Leucopenia defined as leukocyte count $<4 \times 10^9$/L.

with CD19-specific microbeads according to the manufacturer's instructions. Purity was confirmed at >95% by control sampling via flow cytometry (Gallios, Beckman Coulter, Brea, CA, USA). For preparation of total RNA, the RNeasy Mini Kit (Qiagen) was used.

2.3 | Transcriptome profiling

RNA quantity and quality were assessed using Agilent TapeStation 2200 (Agilent, Santa Clara, CA, USA). All RNA samples had RNA integrity number (RIN) values >8. Libraries for transcriptome sequencing were prepared from 1 μg of total RNA using the TruSeq stranded mRNA sample preparation kit including poly-A selection (Illumina, San Diego, CA, USA) followed by paired-end sequencing with 50-bp read length on a HiSeq2500 instrument (Illumina) to an average read depth of 20 M read pairs per sample. Sequencing data were quality controlled using the RNA-SeQC pipeline including assessment of read counts, coverage and cross-sample correlation of expression levels.[16] Alignment of sequencing reads to the human reference genome (build GRCh37) was performed with TopHat2 (2.0.4).[17] For expression analysis on the NanoString nCounter system (NanoString Technologies, Seattle, WA, USA), 50 ng of total RNA was analysed using the Human Immunology v2 CodeSet. Data were quality controlled and normalized using the nSolver Analysis Software v3.0 (NanoString Technologies).

2.4 | Serum-IFN and serum-BAFF analyses

Serum levels of IFN-α were determined with an in-house immunoassay.[18] Serum levels of B-cell activating factor (BAFF)/B Lymphocyte stimulator (BLyS) were determined with the human BAFF-BLys/TNFSF13B Quantikine ELISA kit (R&R Systems, Minneapolis, USA). The cut-off level for IFN-α and BAFF was 0.5 IU/mL and 100 pg/mL, respectively.

2.5 | Statistical analyses

Analysis of differential expression in the RNA-sequencing data set was conducted within the Cufflinks pipeline.[19] Expression levels were normalized to fragments per kilobase of exon per million fragments mapped (FPKM). The quality controlled RNA-sequencing data set comprised

expression levels for >14 000 genes in total, and a false discovery rate (FDR)-corrected significance cut-off of $\alpha < 0.05$ (Q-value) was applied to call differentially expressed genes. For the NanoString data set, differential expression was tested by the Mann-Whitney *U* test (two-sided), and a *P*-value <.05 was considered to be significant.

Functional gene-set enrichment analysis and analysis of subcellular localization of differentially expressed genes were performed for genes with expression fold change (FC) >2 or <0.5, using Ingenuity Pathway Analysis software (Qiagen). Fisher's exact test (two-sided) and a Bonferroni-corrected significance threshold ($P < .00357$) were applied to define differential subcellular localization of differentially expressed genes. IFN-regulated genes were assigned according to the Interferome v2.01 database.[20] Expression values of *IFI35, IFITM1*, *IRF7*, *MX1* and *STAT1* were used to calculate a type I IFN score for each study subject as described.[21] In brief, the mean $(M)_{expr\_ctrl}$ and standard deviation $(SD)_{expr\_ctrl}$ of expression of each gene in the control group were used to derive an expression score for the respective gene in each individual (Expression score = $(Expression_{individual} - M_{expr\_ctrl})/SD_{expr\_ctrl}$). Subsequently, expression scores of the five genes were summed for each individual resulting in a type I IFN score. An individual's IFN score was classified as high if above $M_{score\_ctrl} + 2\ SD_{score\_ctrl.}$[22] Genes involved in epigenetic regulation were characterized according to the EpiFactors database.[23] Unpaired analyses of continuous variables were performed using a Mann-Whitney *U* test with $P < .05$ defining statistical significance. All statistical analyses were conducted in the R environment (v3.4.3, r-project.org/).

3 | RESULTS

3.1 | Differential gene expression in pSS B cells

RNA-sequencing identified major differences in expression levels between CD19+ B cells from anti-SSA–positive patients with pSS compared with healthy control individuals. In total, we observed 4047 autosomal genes that were differentially expressed in pSS B cells. Of these genes, 1826 were overexpressed, and 2221 had lower expression levels in B cells from patients compared with controls (Tables S2 and S3). Principal component analysis of the whole transcriptome data set as well as unsupervised hierarchical clustering of the top 500 differentially expressed genes distinguished clearly between patients with pSS and controls (Figures S1 and S2). We detected numerous IFN-induced genes that displayed higher expression levels in CD19+ B cells from patients compared with controls (FC > 2, $P \leq 5 \times 10^{-5}$). These genes encompassed both type I (IFN-α)-induced genes, such as *CX3CR1*, *TNFSF4*, *TNFSF10*, *S100A8/A9*, *IFI27*, *IFI44L*, *IFI44*, *MX1*, *PARP9*, *OAS1* and *OAS2*, as well as type II (IFN-γ)-induced genes, such as *GBP1*, *GBP5* and *IFI16*, establishing a type I and type II IFN signature in pSS B cells (Table 2; Figures 1 and 2; Table S2; Figures S3 and S4).

Decreased expression of multiple members of suppressors of cytokine signalling (SOCS) via JAK1 and JAK2, such as *SOCS1* and *SOCS3*, was revealed in pSS B cells compared with controls (FC 0.4 and 0.1, respectively, both $P \leq 5 \times 10^{-5}$). Additional genes implicated in negative regulation of cytokine signalling which was downregulated in pSS B cells included *PTPN1* (FC 0.5, $P \leq 5 \times 10^{-5}$) and *SH2B3* (FC 0.3, $P \leq 5 \times 10^{-5}$). PTPN1 acts by dephosphorylating JAK and TYK kinases, and the *SH2B3* gene, previously associated with multiple autoimmune disorders including RA, type 1 diabetes, celiac disease and SLE,[24-26] is another key regulator of cytokine signalling. Of note, *TNFAIP3*, encoding A20, a negative regulator of NFκB signalling, was also prominently downregulated in B cells from patients with pSS (FC 0.2, $P \leq 5 \times 10^{-5}$) (Figure 1 and Table S3).

Investigating differential expression of non-HLA loci with genetic variants that have been associated with pSS susceptibility in Caucasians,[27] we found decreased expression of *CXCR5*, *DDX6* and *IL12A* in patients with pSS. Differential expression in pSS B cells was not detected for *BLK*, *FAM167A*, *STAT4, TNIP1, IRF5* or *TNPO3* (Table S4).

3.2 | X-chromosomal differential expression

In the analysis of X-chromosomal gene expression, 12 female patients with pSS and 16 female control individuals were included. Differential expression was observed for 153 genes, of which several were implicated in immune system signalling such as Toll-like receptors 7 and 8 (*TLR7*, *TLR8*), which were upregulated in pSS B cells (FC 2.8 and 9.3, respectively, both $P \leq 5 \times 10^{-5}$), as well as Bruton tyrosine kinase (*BTK*), which has a role in B cell receptor signalling (FC 2.0, $P \leq 5 \times 10^{-5}$). Among downregulated genes, we noted TSC22 domain family member 3 (*TSC22D3*), involved in anti-inflammatory immune responses upon interleukin (IL)-10 stimulation (FC 0.5, $P = 4.5 \times 10^{-4}$) (Table S5 and Figure S5).

3.3 | Pathway analysis and subcellular location of differentially expressed genes

Autosomal genes that were differentially expressed were further subjected to functional pathway analysis. Glucocorticoid receptor signalling ($P = 1.2 \times 10^{-8}$), role of pattern recognition receptors in recognition of bacteria and viruses

TABLE 2 Genes with validated upregulated expression in B cells from anti-SSA–positive patients with pSS compared with controls (expression fold change ≥2.0 by RNA-sequencing)

				RNA-sequencing[a]					Validation[a]	
Gene symbol	Gene name	Chr	IFN regulated[b]	FPKM pSS	FPKM ctrl	FC[c]	*P*-value[d]	Q-value[e]	FC	*P*-value[d]
CX3CR1	C-X3-C motif chemokine receptor 1/Fractalkine receptor	3	Yes	4	0.3	15.8	5.0×10^{-5}	3.8×10^{-4}	2.6	7.7×10^{-3}
TNFSF4	Tumour necrosis factor superfamily member 4/ Ox40L	1	Yes	5	0.4	10.6	5.0×10^{-5}	3.8×10^{-4}	1.9	7.8×10^{-4}
TNFSF10	Tumour necrosis factor superfamily member 10/ TRAIL	3	Yes	25	4	6.5	5.0×10^{-5}	3.8×10^{-4}	1.7	4.5×10^{-5}
CCL5	C-C motif chemokine ligand 5/RANTES	17	Yes	42	7	5.7	5.0×10^{-5}	3.8×10^{-4}	2.1	.012
S100A8	S100 calcium binding protein A8/Calgranulin A	1	Yes	96	21	4.7	5.0×10^{-5}	3.8×10^{-4}	1.8	.025
MX1	MX dynamin-like GTPase 1	21	Yes	146	34	4.3	5.0×10^{-5}	3.8×10^{-4}	3.8	4.7×10^{-7}
GBP1	Guanylate binding protein 1	1	Yes	14	3	4.2	5.0×10^{-5}	3.8×10^{-4}	2.5	4.6×10^{-6}
S100A9	S100 calcium binding protein A9/Calgranulin B	1	Yes	272	67	4.1	5.0×10^{-5}	3.8×10^{-4}	1.7	.041
IL32	Interleukin 32	16	Yes	2	0.6	3.9	5.0×10^{-5}	3.8×10^{-4}	1.5	2.1×10^{-4}
TNFRSF17	TNF receptor superfamily member 17/BCMA	16	–	28	7	3.8	5.0×10^{-5}	3.8×10^{-4}	1.5	1.9×10^{-3}
TNFSF13B	Tumour necrosis factor superfamily member 13b/ BAFF	13	Yes	6	2	3.8	5.0×10^{-5}	3.8×10^{-4}	2.2	6.7×10^{-4}
CCR1	C-C motif chemokine receptor 1	3	Yes	3	1	3.6	5.0×10^{-5}	3.8×10^{-4}	1.6	7.7×10^{-3}
STAT1	Signal transducer and activator of transcription 1	2	Yes	102	29	3.5	5.0×10^{-5}	3.8×10^{-4}	2.7	3.3×10^{-7}
IFI35	Interferon-induced protein 35	17	Yes	28	10	2.9	5.0×10^{-5}	3.8×10^{-4}	2.2	4.7×10^{-7}
IRF7	Interferon regulatory factor 7	11	Yes	62	22	2.8	5.0×10^{-5}	3.8×10^{-4}	2.4	6.4×10^{-7}
TLR7	Toll-like receptor 7	X	Yes	18	6	2.8	5.0×10^{-5}	3.8×10^{-4}	2.1	4.5×10^{-7}
ZAP70	Zeta chain of T cell receptor associated protein kinase 70	2	Yes	7	3	2.5	5.0×10^{-5}	3.8×10^{-4}	1.5	0.023
ICAM2	Intercellular adhesion molecule 2	17	Yes	47	20	2.4	5.0×10^{-5}	3.8×10^{-4}	1.2	.014
BST2	Bone marrow stromal cell antigen 2	19	Yes	65	28	2.3	5.0×10^{-5}	3.8×10^{-4}	1.8	9.7×10^{-6}
LILRB2	Leukocyte immunoglobulin-like receptor B2/ILT4	19	–	5	2	2.3	5.0×10^{-5}	3.8×10^{-4}	1.9	8.1×10^{-4}
MS4A1	Membrane spanning 4-domains A1/CD20	11	Yes	497	225	2.2	5.0×10^{-5}	3.8×10^{-4}	1.2	5.7×10^{-4}
BTLA	B and T lymphocyte associated	3	–	87	39	2.2	5.0×10^{-5}	3.8×10^{-4}	1.3	2.2×10^{-3}
CR2	Complement C3d receptor 2	1	Yes	30	14	2.2	5.0×10^{-5}	3.8×10^{-4}	1.3	.011
STAT2	Signal transducer and activator of transcription 2	12	Yes	66	30	2.2	5.0×10^{-5}	3.8×10^{-4}	1.8	7.7×10^{-8}

(Continues)

TABLE 2 (Continued)

Gene symbol	Gene name	Chr	IFN regulated[b]	RNA-sequencing[a] FPKM pSS	FPKM ctrl	FC[c]	*P*-value[d]	Q-value[e]	Validation[a] FC	*P*-value[d]
GBP5	Guanylate binding protein 5	1	Yes	17	8	2.1	5.0×10^{-5}	3.8×10^{-4}	2.0	6.7×10^{-3}
IFI16	Interferon gamma-inducible protein 16	1	Yes	80	37	2.1	5.0×10^{-5}	3.8×10^{-4}	1.5	1.7×10^{-7}
CYBB	Cytochrome b-245 beta chain	X	Yes	135	67	2.1	5.0×10^{-5}	3.8×10^{-4}	1.2	5.6×10^{-5}
BTK	Bruton tyrosine kinase	X	–	110	54	2.0	5.0 × 10-5	3.8 × 10-4	1.2	3.8 × 10-5

FPKM, fragments per kilobase of exon per million fragments mapped; FC, expression fold change.

[a]Analysed by RNA-sequencing (for autosomal genes in n = 12 pSS, n = 20 controls; for X-chromosomal genes in n = 12 female pSS, n = 16 female controls), validation cohort (for all genes in n = 17 female pSS, n = 16 female controls) analysed by a NanoString gene expression panel.

[b]Database of interferon-regulated genes http://interferome.org.

[c]Differential gene expression as FC of mean FPKM between patients with pSS and controls.

[d]Uncorrected *P*-value of differential gene expression between patients with pSS and controls.

[e]False discovery rate (FDR) < 0.05 corrected significance for differential gene expression between patients with pSS and controls.

($P = 1.2 \times 10^{-8}$), activation of IRFs by cytosolic pattern recognition receptors ($P = 1.6 \times 10^{-8}$), IFN signalling ($P = 3.2 \times 10^{-8}$) and TLR signalling ($P = 6.7 \times 10^{-8}$) were the most significantly enriched canonical pathways in our data set (Table S6).

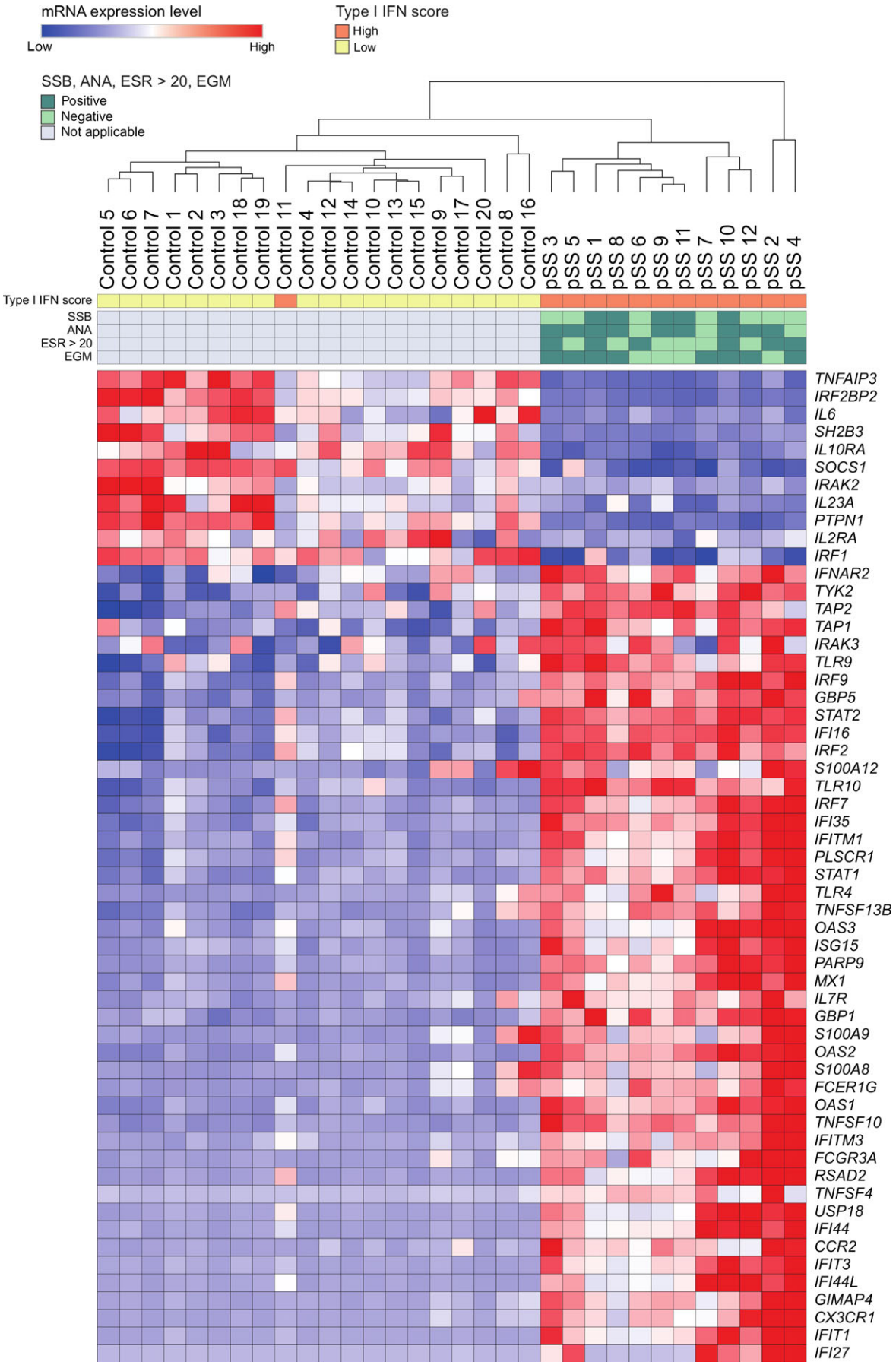
Next, we investigated the subcellular localization of differentially expressed genes using gene annotation data from the Ingenuity Pathway Analysis tool. Genes with increased expression in pSS B cells were enriched in the extracellular space ($P = 4.0 \times 10^{-4}$), while genes with decreased expression were more often located in the nucleus ($P < 1.0 \times 10^{-4}$) (Figure S6a). These distinct local enrichments correspond to the finding that upregulated genes were more commonly transmembrane receptors ($P < 1.0 \times 10^{-4}$), while downregulated genes were more commonly regulators of transcription ($P < 1.0 \times 10^{-4}$) and translation ($P = .001$) (Figure S6b).

3.4 | Validation of differential gene expression

Differential expression in CD19+ B cells from a validation cohort consisting of 17 anti-SSA–positive female patients with pSS and 16 female controls was analysed by the NanoString Human Immunology v2 gene expression panel. Of the 4200 differentially expressed genes (both autosomal and X-chromosomal) defined by RNA-sequencing, 164 genes were covered by the validation panel. Of these, 57 genes (34.8%) were also differentially expressed by NanoString, 52 upregulated and 5 downregulated in pSS B cells (Tables 2 and 3 and Table S7). The upregulated gene displaying the largest FC by RNA-sequencing and validated by NanoString was the chemokine receptor gene *CX3CR1* encoding the fractalkine receptor (Figure 2A). Several genes belonging to the tumour necrosis factor superfamily (TNFSF), namely *TNFSF4* encoding Ox40 ligand (Ox40L) (Figure 2B), *TNFSF10* encoding TNF-related apoptosis-inducing ligand (TRAIL) (Figure 2C), *TNFSF13B* encoding BAFF and *TNFRSF17* encoding the BAFF-receptor B-cell maturation antigen (BCMA), were among the validated upregulated genes in pSS B cells.

In addition to the type I IFN-induced gene *MX1* (Figure 2D), other validated genes within the IFN signalling pathway with an upregulated expression in pSS B cells were signal transducer and activator of transcription 1 and 2 (*STAT1*, *STAT2*). We calculated a type I IFN score for each study individual and found a distinctly elevated type I IFN score in all patients with pSS analysed by RNA-sequencing and in 15 of 17 patients in the validation cohort, both $P < 1 \times 10^{-5}$ compared with healthy controls

FIGURE 1 Differential expression of interferon (IFN)-regulated genes in anti-SSA–positive pSS B cells. Heat map based on unsupervised hierarchical clustering of the expression levels of 56 IFN-regulated genes in B cells from patients with pSS (n = 12) and controls individuals (n = 20) as determined by RNA-sequencing. Each column represents one sample. Type I IFN scores for each individual are represented in the upper panel, with "high" indicated in orange (defined as a score above mean $(M)_{score\ controls}$ + 2 standard deviation $(SD)_{score\ controls}$) and "low" indicated in yellow. The middle panel illustrates clinical characteristics of the individuals, with "positive" indicated in dark green, "negative" in light green and "not applicable" in grey. The heat map in the lower panel illustrates gene expression levels, and each row represents one IFN-regulated gene. The colour scale of the expression heat map is based on z-score distribution of gene expression levels, from −2 (blue) to 2 (red). SSB, anti-SSB antibody; ANA, antinuclear antibody; ESR > 20, erythrocyte sedimentation rate >20 (mm/h); EGM, extraglandular manifestations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High
Type I IFN score
High
Low
SSB, ANA, ESR > 20, EGM
Positive
Negative
Not applicable
Control 5
Control 6
Control 7
Control 1
Control 2
Control 3
Control 18
Control 19
Control 11
Control 4
Control 12
Control 14
Control 10
Control 13
Control 15
Control 9
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Type I IFN score
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TNFAIP3
IRF2BP2
IL6
SH2B3
IL10RA
SOCS1
IRAK2
IL23A
PTPN1
IL2RA
IRF1
IFNAR2
TYK2
TAP2
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TLR9
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GBP5
STAT2
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PLSCR1
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OAS1
TNFSF10
IFITM3
FCGR3A
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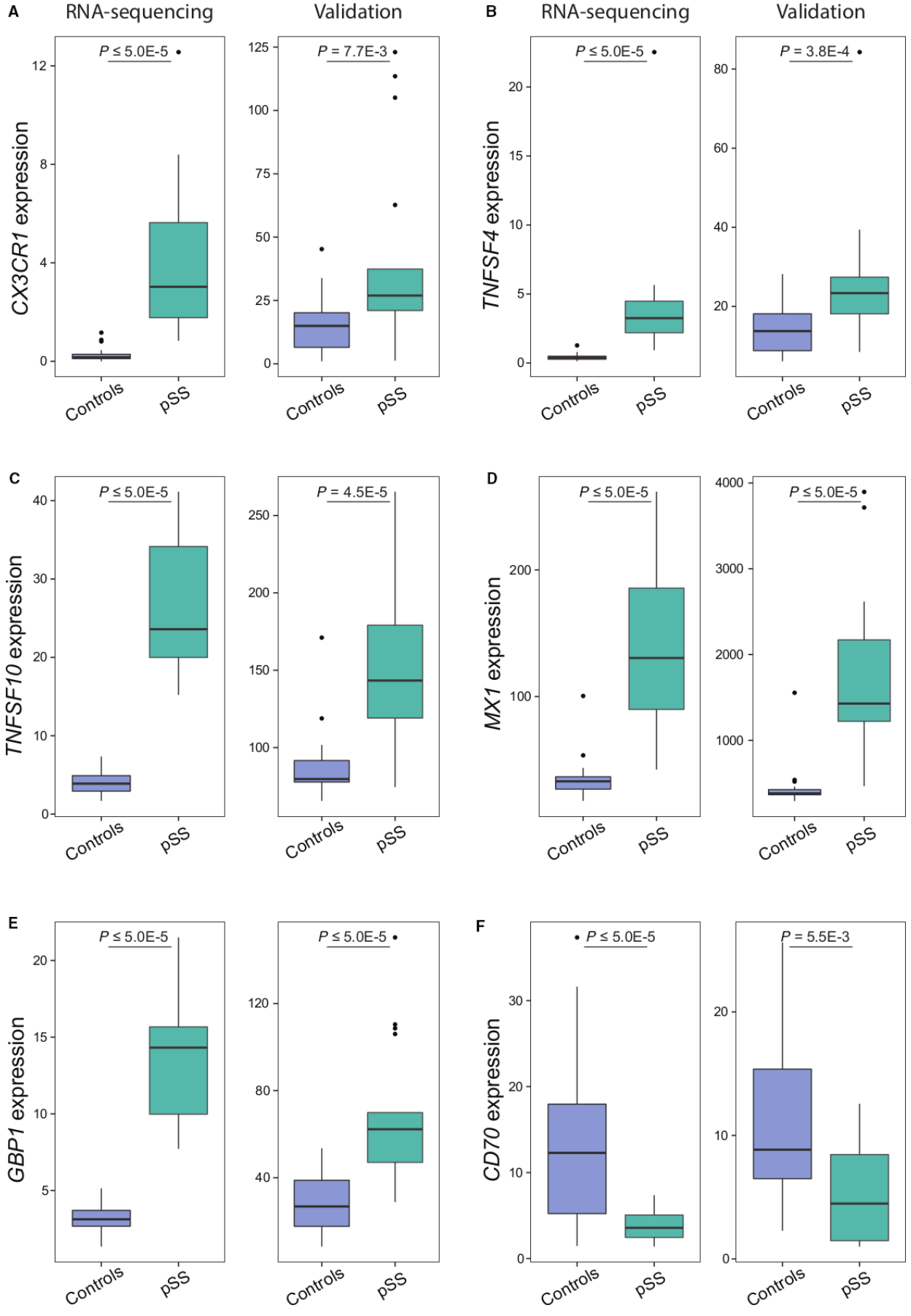
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RNA-sequencing
Validation
CX3CR1 expression
P ≤ 5.0E-5
P = 7.7E-3
B
RNA-sequencing
Validation
TNFSF4 expression
P ≤ 5.0E-5
P = 3.8E-4
C
TNFSF10 expression
P ≤ 5.0E-5
P = 4.5E-5
D
MX1 expression
P ≤ 5.0E-5
P ≤ 5.0E-5
E
GBP1 expression
P ≤ 5.0E-5
P ≤ 5.0E-5
F
CD70 expression
P ≤ 5.0E-5
P = 5.5E-3
Controls
pSS

FIGURE 2 Gene expression levels in B cells from controls and anti-SSA–positive patients with pSS. Boxplots representing mRNA expression levels determined by RNA-sequencing (left panels: controls n = 20; pSS n = 12) and validation (right panels: controls n = 16; pSS n = 17) of (A) *CX3CR1*, (B) *TNFSF4*, (C) *TNFSF10*, (D) *MX1*, (E) *GBP1* and (F) *CD70*

(Figure S3).The type II (IFNγ)-induced genes *GBP1* (Figure 2E), *GBP5* and *IFI16* were also overexpressed in both data sets, validating a type I and type II IFN signature in pSS B cells (Figure S4). Further notable genes with validated upregulated expression in pSS B cells were the chemokine ligand *CCL5* (RANTES) and one of its receptors, *CCR1*. The downregulated gene displaying the largest FC as identified by RNA-sequencing and validated by NanoString was *CD70*, alias *TNFSF7*, encoding the CD70 molecule present on activated T and B lymphocytes (Figure 2F).

While *TLR7*, *TLR8* and *TLR9* displayed upregulated expression in pSS B cells by RNA-sequencing, only *TLR7* was also significantly overexpressed in the validation cohort (Table 2; Tables S2 and S5). However, *TLR8* expression was non-significantly upregulated in the validation cohort (FC 2.0, $P = .14$), with no differential expression seen for *TLR9*. Sera sampled simultaneously as B cells were available from the validation cohort. Both IFN-α and BAFF serum levels were significantly elevated in pSS compared with controls ($P = .011$ and $P = .0077$, respectively). A correlation between *TNFSF13B* (BAFF) mRNA expression and serum-BAFF levels was found in patients with pSS ($r = .66$, $P = .0042$), but not in controls (Figure S7).

3.5 | Gene expression of differentially methylated genes and epigenetic regulator genes

In a previous study, we performed whole-genome DNA methylation profiling in primary CD19+ B cells from 24 patients with pSS and 47 healthy controls and identified 453 differentially methylated autosomal CpG sites annotated to 303 unique genes in pSS.[5] The patients with pSS and controls, subjected to RNA-sequencing in this study, constitute a subset of the individuals included in our previous study, and methylation data were available from all individuals. Of the 4047 autosomal genes that were differentially expressed in pSS B cells, DNA methylation data were available for 3440 genes. Of these, 77 genes were identified as differentially methylated in pSS B cells applying a stringent significance threshold of $P < 1.3 \times 10^{-7}$ to define differential methylation (Bonferroni correction) (Table S8). For X-chromosomal genes, DNA methylation was assessed in 24 female patients with pSS and 35 female controls. Intersection with expression data generated by RNA-sequencing revealed differential methylation at 21 CpG sites annotated to 16 unique X-chromosomal differentially expressed genes in pSS B cells (Table S9). Among genes with both differential expression and differential methylation, we identified central mediators of innate and adaptive immune system signalling, such as *C2*, *CXCR5*, *IL23A*, *TLR7* and *TNFSF4*, and several IFN-signature genes, such as *MX1*, *IFI44L*, *PARP9*, *IFIT1*, *IFITM1*, *IFITM3* and *RSAD2* (Figure 3; Tables S8 and S9).

Investigating the expression of genes involved in epigenetic processes as defined according to the EpiFactors database,[23] we identified 123 differentially expressed epigenetic factor genes in pSS B cells. Among genes with upregulated expression in pSS were male-specific lethal 3 homolog (*MSL3*) and histone deacetylase 6 (*HDAC6*) (FC 1.7 and

TABLE 3 Genes with validated downregulated expression in B cells from anti-SSA–positive patients with pSS compared with controls

				RNA-sequencing[a]					Validation[a]	
Gene symbol	Gene name	Chr	IFN regulated[b]	FPKM pSS	FPKM ctrl	FC[c]	*P*-value[d]	Q-value[e]	FC	*P*-value[d]
CD70	CD70 molecule/TNFSF7	19	–	4	14	0.3	5.0×10^{-5}	3.8×10^{-4}	0.6	5.5×10^{-3}
TCF7	Transcription factor 7	5	–	6	13	0.5	5.0×10^{-5}	3.8×10^{-4}	0.7	.023
IL23A	Interleukin 23 subunit alpha	12	Yes	3	5	0.5	3.5×10^{-4}	2.1×10^{-3}	0.6	2.9×10^{-3}
IL2RA	Interleukin 2 receptor subunit alpha	10	Yes	3	5	0.5	1.9×10^{-3}	8.7×10^{-3}	0.6	4.9×10^{-4}
CD82	CD82 molecule	11	Yes	38	50	0.8	5.6×10^{-3}	0.022	0.8	7.7×10^{-3}

FPKM, fragments per kilobase of exon per million fragments mapped; FC, expression fold change.

[a]Analysed by RNA-sequencing (n = 12 pSS, n = 20 controls), validation cohort (n = 17 pSS, n = 16 controls) analysed by a NanoString gene expression panel.

[b]Database of interferon-regulated genes http://interferome.org.

[c]Differential gene expression as FC of mean FPKM between patients with pSS and controls.

[d]Uncorrected *P*-value of differential gene expression between patients with pSS and controls.

[e]False discovery rate (FDR) < 0.05 corrected significance for differential gene expression between patients with pSS and controls.

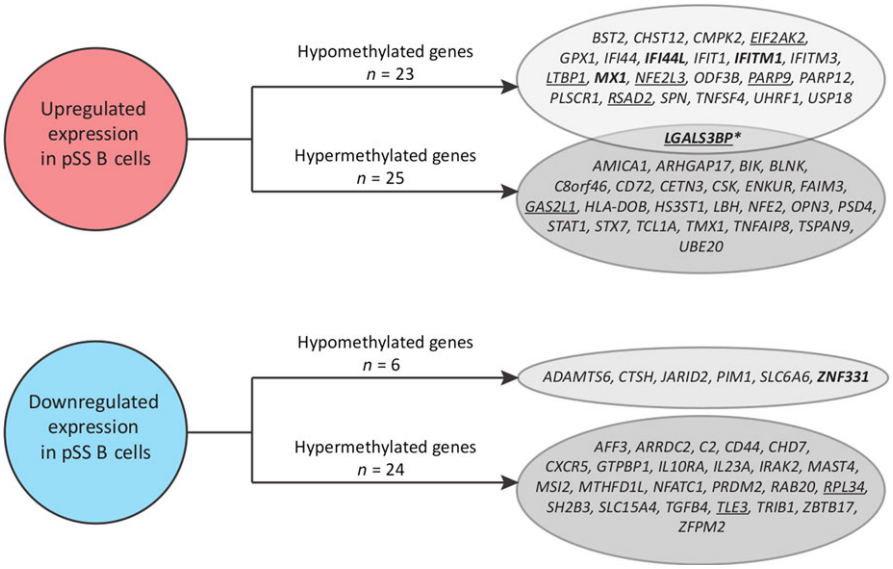

FIGURE 3 Overlap of differential gene expression and differential DNA methylation in pSS B cells. Autosomal genes identified as both differentially expressed by RNA-sequencing (pSS n = 12; controls n = 20) and differentially methylated (pSS n = 24; controls=47) by HumanMethylation450k BeadChip arrays in CD19+ B cells from patients with pSS compared with control individuals are presented. The individuals in the gene expression analysis constitute a subset of the individuals in the methylation study. Underlined gene symbols indicate genes containing two differentially methylated CpG sites, and gene symbols in bold indicate genes containing three or more differentially methylated CpG sites. *For LGALS3BP, four hypo- and two hypermethylated CpG sites were identified in pSS B cells

1.6, respectively, both $P \leq 5 \times 10^{-5}$), involved in histone acetylation and deacetylation, and deltex E3 ubiquitin ligase 3L (*DTX3L*) (FC 2.5, $P \leq 5 \times 10^{-5}$), which plays a central role in histone ubiquitination. For lysine demethylase 6A and 6B (*KDM6A* and *KDM6B*) (FC 0.7 and 0.2, $P = 1.5 \times 10^{-4}$ and $P \leq 5 \times 10^{-5}$, respectively), which act in histone demethylation processes, we found decreased expression levels in B cells from patients with pSS (Tables S2, S3 and S5).

4 | DISCUSSION

B cell hyperactivity is a key feature in pSS with autoantibody production, antigen presentation and secretion of cytokines, both systemically and locally in the minor salivary glands.[1] To our knowledge, this is the first study to investigate the whole CD19+ B cell transcriptome in antibody-positive pSS using RNA-sequencing. We found widespread deregulation of gene expression in pSS B cells, including upregulation of several key genes involved in B cell activation, T cell costimulation, chemokine and pro-inflammatory cytokine secretion, while negative regulators of immune activation were downregulated, schematically summarized in Figure 4.

The top upregulated and validated gene was the chemokine receptor *CX3CR1*, encoding the fractalkine receptor expressed on lymphocytes, mainly T cells, monocytes and NK cells, as well as on smooth muscle cells, neurons and tumour cells. Its ligand, fractalkine is encoded by the *CX3CL1* gene and promotes migration and adhesion of inflammatory cells to the endothelium and epithelium.[28] Here, we demonstrate increased mRNA expression of *CX3CR1* in peripheral CD19+ B cells from patients with pSS. Upregulated expressions of *CX3CL1*/fractalkine protein on ductal epithelial cells and *CX3CR1*/fractalkine receptor on inflammatory cells in lymphocyte foci, primarily in germinal centre-like formations, have been demonstrated in salivary glands from patients with pSS.[29] Interestingly, a role for the CX3CL1/CX3CR1 axis in B cell malignancies has been proposed. While B cells normally do not express *CX3CR1*, B cell lymphomas of different subtypes including marginal zone lymphomas of mucosa-associated lymphoid tissue (MALT), the most common lymphoma subtype in pSS, express *CX3CR1* at both the mRNA and protein levels. This is thought to facilitate the interaction between lymphoma cells and the microenvironment.[28] While none of our patients had a history of lymphoma, the upregulation of *CX3CR1* as presented here may indicate that B cells from patients with pSS are primed for immune activation and possible lymphoma transformation.

Another chemokine receptor with upregulated expression in pSS B cells was *CCR1* together with one of its ligands *CCL5*/RANTES. Lisi et al[30] reported upregulated expression of NFκB pro-inflammatory target genes, including *CCR1*, by salivary gland epithelial cells treated with anti-SSA antibodies. Szodoray et al[31] found increased serum levels of CCL5 only in those patients with pSS

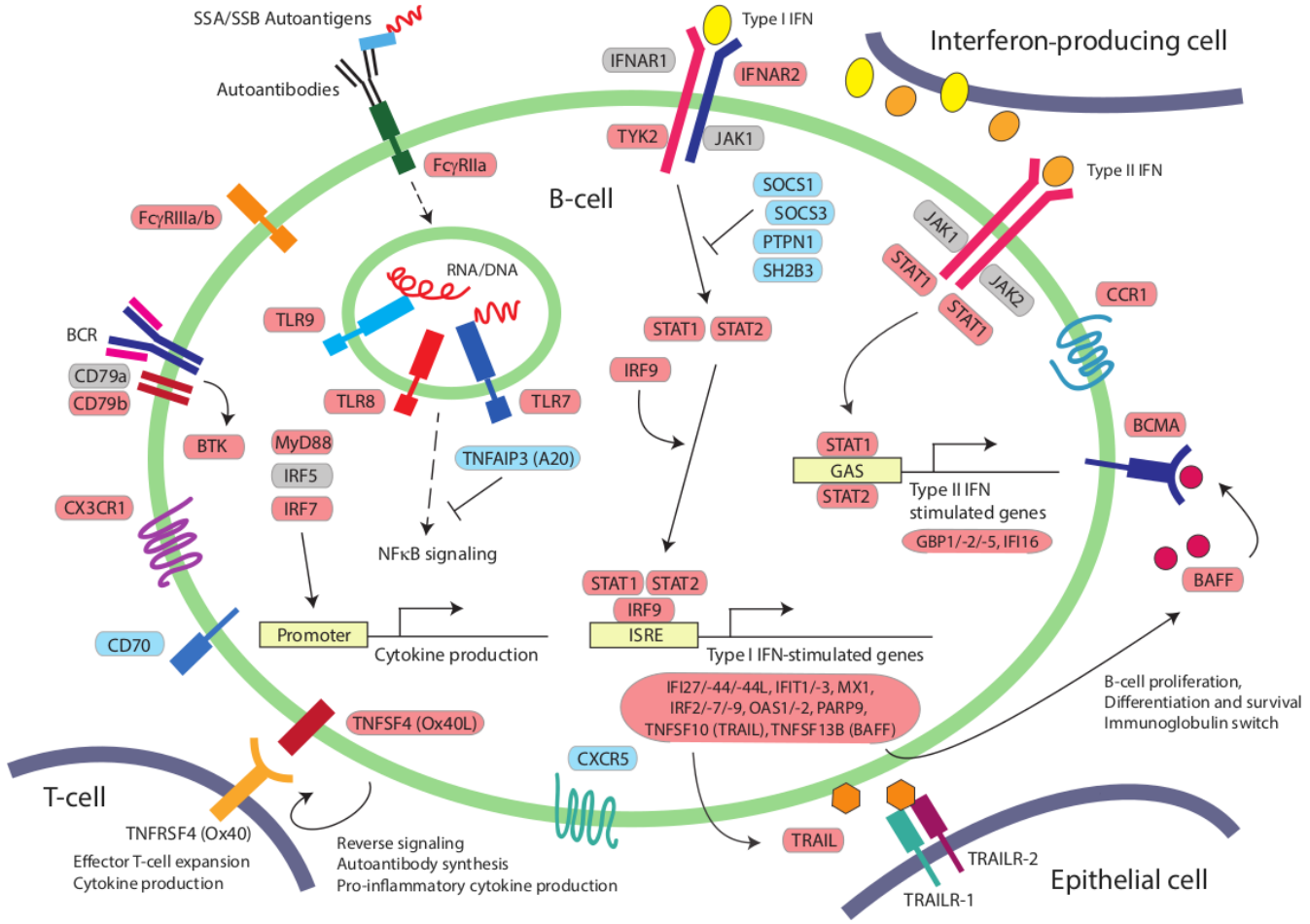

FIGURE 4 Schematic illustration of differential gene expression in pSS B cells analysed by RNA-sequencing. Genes with increased expression in CD19+ B cells of anti-SSA–positive patients with pSS compared with controls are indicated in red, genes with decreased expression in pSS patients are indicated in blue, and genes not differentially expressed between cases and controls are indicated in grey

presenting with a high erythrocyte sedimentation rate (ESR), as a reflection of B cell activation and hypergammaglobulinemia. This is in concordance with the results from our study, where we selected pSS patient positive for SSA antibodies, and about half of the patients had an ESR of >20 mm/h, underlining the role of upregulated chemokines and chemokine receptors for immune cell migration in antibody-positive patients with pSS.

Further, we note two members of the TNF superfamily among the top upregulated and validated genes, *TNFSF4*/Ox40L and *TNFSF10*/TRAIL. Ox40L has a role in promoting the expansion of T follicular helper cells (Tfh), which are essential for germinal centre-like formation.[32,33] Reverse signalling through Ox40L can contribute to B cell differentiation and systemic autoantibody production as seen in pSS.[32] We have previously demonstrated that genetic polymorphisms in *TNFSF4* are associated with risk for pSS,[34] but this is to our knowledge the first time an increased mRNA expression of *TNFSF4* is demonstrated in any cell type in pSS. A study of pSS minor salivary glands demonstrated TRAIL protein expression on infiltrating mononuclear cells and TRAIL-receptors on ductal epithelial cells, which may indicate a role for the TRAIL system in regulating autoimmunity in pSS salivary glands.[35] The upregulated expression of *TNFSF10* mRNA found in this study suggests a regulatory role for TRAIL in the autoimmune process not only in the salivary glands, but also in pSS B cells.

Other upregulated and validated genes within the TNF superfamily were *TNFSF13B*/BAFF and its receptor *TNFRSF17*/BCMA. BAFF is central for B cell proliferation, survival and immunoglobulin switch, and serum levels of BAFF are increased in pSS, correlating with autoantibody levels, disease activity and increased risk for lymphoproliferative disorder.[36-38] Increased *BAFF* and *BCMA* mRNA and BAFF protein expression have been observed in minor salivary glands from patients with pSS.[10,39] Here, we demonstrate upregulated expression of *TNFSF13B* in pSS B cells, correlating with increased BAFF serum levels, further supporting the notion that an IFN signature with upregulated *TNFSF13B* contributes to B cell activation in pSS.

Genes encoding multiple S100 proteins, including *S100A8/S100A9* (calgranulin A/B, also known as

calprotectin), were upregulated in pSS B cells. Serum levels of calprotectin have been suggested as biomarkers of rheumatic diseases, and in inflammatory bowel disease faecal levels of calprotectin are in clinical use for assessing inflammation.[40] Increased levels of calprotectin have been found in sera and salivary glands from patients with pSS and elevated faecal levels were detected in pSS with concomitant gastrointestinal disease.[41,42] To our knowledge, this is the first time increased gene expression of *S100A8/S100A9* in pSS B cells has been demonstrated, and the possible impact on systemic inflammation in pSS warrants further investigation.

Further, we established a prominent type I and type II IFN signature in pSS B cells. Type I IFN has pleiotropic activating effects on the immune system, and IFN signalling was among the top differentially expressed pathways in our data set. An etiopathogenic mechanism is that endogenous immune complexes consisting of nucleic acid-binding proteins and their autoantibodies, for example anti-SSA/SSB, bind to Fcγ-receptor IIA (CD32) before being internalized into endosomes for ligation to TLR7, ultimately leading to IFN production.[43] We observed upregulation of *FCGR2A*, *TLR7* and the downstream signalling molecules *MYD88* and *IRF7* in pSS B cells (Table S7). Type I IFNs bind to the type I IFN receptor (IFNAR) and signal via STAT1 and STAT2, which showed increased mRNA expression in our study, to elicit the expression of type I IFN-induced genes, that is the IFN signature.[44] On the contrary, *SOCS1* and *SOCS3*, which play a central role in downregulation of type I IFN pathway signalling, showed a decreased expression in pSS B cells, possibly permitting a more pro-inflammatory state in pSS compared with controls. Several studies indicate that the IFN signature in pSS primarily can be observed in patients positive for anti-SSA/SSB antibodies, and in our current study, only antibody-positive patients were included.[9,45] There is a considerable overlap between type I and II IFN-induced genes, and the impact of IFN-γ in the pathogenesis of pSS is yet to be determined.[22]

TLR7 and TLR8 are phylogenetically similar endosomal receptors, recognizing viral and bacterial RNA as well as oligoribonucleotides. B cells from healthy controls express *TLR7* mRNA, while *TLR8* expression is absent or only detected at low levels.[46] Overexpression of *TLR7* in different cell types from patients with pSS has been described.[9,11] Here, we demonstrate upregulated expression of both *TLR7* and *TLR8* on pSS B cells, possibly induced by nucleic acid-containing immune complexes present in anti-SSA/SSB-positive pSS.

Genes and signalling pathways involved in the transition from benign activated polyclonal B cells to pSS B cell lymphomas are only partly understood.[47] Interestingly, BTK inhibitors are used for treatment of B cell malignancies, and we observed upregulation of *BTK* mRNA in pSS B cells.[48] In concordance with our results, a recent study revealed upregulated BTK protein expression in stimulated B cells from the majority of patients with pSS.[49] However, a link between BTK upregulation and lymphomagenesis in pSS has not yet been determined. On the other hand, genetic variants in *TNFAIP3*, encoding A20, a negative regulator of TNF- and TLR-stimulated activation of NF-κB signalling, have been linked to lymphoma in pSS.[50,51] Here, we detected downregulated *TNFAIP3* expression, allowing a pro-inflammatory state in pSS B cells. The differential expression of genes involved in lymphomagenesis in B cells from antibody-positive patients with pSS as here presented is intriguing and justifies further exploration.

The impact of epigenetic regulation of gene expression is only partly understood. While the most strongly hypomethylated genes from our previous study showed upregulated expression according to RNA-sequencing,[5] the pattern of inverse correlation between DNA methylation and gene expression was less clear for other genes. However, we identified promotor region hypermethylation of *CXCR5* and *IL23A* as associated with decreased gene expression in patients with pSS. To fully disentangle the impact of DNA methylation on gene expression in pSS, further studies analysing relevant tissues in large well-characterized cohorts and integration of genetic and additional epigenetic data, such as histone modification marks, are required. In addition, the role of microRNAs (miRNAs) in the pathogenesis of pSS needs to be taken into account, as suggested by a recent study reporting deregulation of miRNA expression in pSS lymphocytes.[52] The aberrant expression of several epigenetic key regulatory factors in pSS B cells as demonstrated here is striking, and its underlying mechanisms and functional implications may be addressed in future studies.

In our study, we included only anti-SSA antibody-positive, untreated female patients with pSS. Despite an apparently inactive disease, patient B cells displayed a transcriptome which was clearly distinguished from that of healthy controls. We also validated the differential expression of important genes in B cells in an independent cohort using an independent method. Nevertheless, limitations of our study are the relatively low number of individuals and the limited number of differentially expressed genes assessed by the validation panel. Peripheral B cell subsets are altered in pSS with lower frequency of memory B cells and increased proportion of naïve B cells compared with controls, which may be reflected in the transcriptomic data.[53] The transcriptome of B cells from men with pSS, autoantibody-negative patients or patients undergoing treatment with immunosuppressive drugs has not yet been explored.

Our results emphasize the central role for B cells in the pathophysiology of pSS, strengthening the concept of B cells as potential therapeutic targets.[54] Trials with B cell depleting antibodies (rituximab) and anti-BAFF/BLyS

antibodies (belimumab) abating B cell activation have shown promising results, primarily in reducing systemic activity in pSS.[55,56] The prominent upregulation of genes involved in type I IFN production and signalling found in this study encourages interfering with specific molecules directed against TLR7, IFN-α, IFNAR and the JAK-STAT pathway. Clinical trials with anifrolumab, a monoclonal antibody targeting the IFNAR, are currently underway in pSS.[54]

In conclusion, we here present whole transcriptome profiling in B cells from patients with autoantibody-positive pSS identifying extensive differences in expression patterns compared with controls, including a prominent type I and type II IFN signature. The impact of increased *CX3CR1* and *TNFSF4*/Ox40L expression for germinal centre-like formations in the salivary glands and for lymphomagenesis warrants further investigations. Our study adds to the current knowledge of the importance of B cells in pSS and as future therapeutic targets.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHORS' CONTRIBUTIONS

JIK, JKS, GN, ACS and MWH conceived and designed the study. AB, MK, GN, MLE, LR and MWH managed recruitment of patients and controls. JIK, JKS, AB and JN analysed the data. JIK and GN wrote the first draft the manuscript, and all authors approved the final version.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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