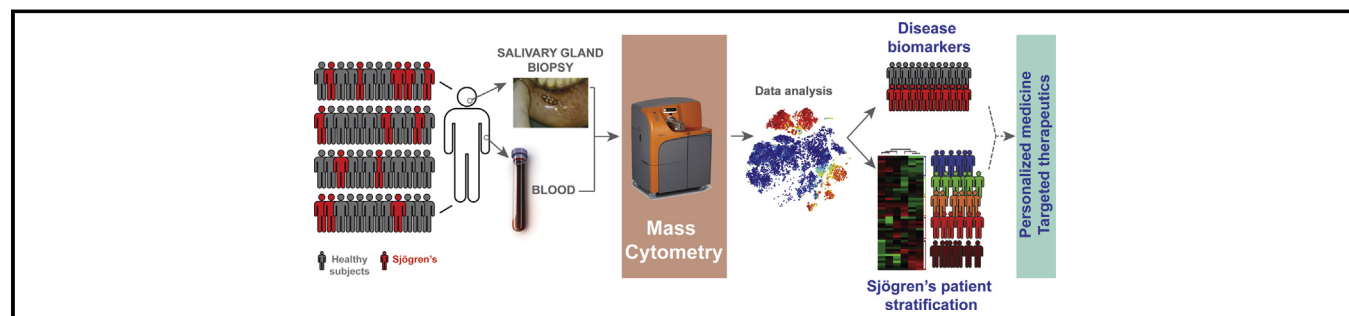


Cytometry by time-of-flight immunophenotyping identifies a blood Sjögren's signature correlating with disease activity and glandular inflammation

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GRAPHICAL ABSTRACT



Background: Mass cytometry has recently emerged as a promising tool for clinical research. However, few studies have demonstrated its benefit for patient stratification and biomarker identification. Primary Sjögren's syndrome (pSS) is a prototype of chronic autoimmune disease, the pathogenesis of which remains unclear and for which treatment does not exist.

Objective: This observational case-control study was designed to discover new cellular biomarkers and therapeutic targets in patients with pSS.

Methods: Forty-nine patients with pSS and 45 control subjects were enrolled for clinical evaluation and mass cytometry quantification of 34 protein markers in blood. For a third of these subjects, matched labial salivary gland biopsy specimens

were also analyzed by mass cytometry and immunohistochemistry.

Results: In salivary gland biopsy specimens from patients with pSS, we identified a high number of activated CD8⁺ T cells, terminally differentiated plasma cells, and activated epithelial cells, pointing to new pathogenic mechanisms for future clinical intervention. In blood, we identified a 6-cell disease signature defined by decreased numbers of CD4 and memory B lymphocytes, decreased plasmacytoid dendritic cell numbers, and increased representation of activated CD4 and CD8 T cells and plasmablasts. These blood cellular components correlated with clinical parameters and, when taken together, clustered patients into subsets with distinct disease activity and glandular inflammation.

Conclusion: This first application of mass cytometry to a well-stratified clinical cohort and small biopsy tissues establishes the benefits of such an approach for the discovery of new biomarkers and therapeutic targets. Similar high-dimensional immunophenotyping strategies could be implemented in longitudinal and interventional clinical settings in this and other disease areas. (*J Allergy Clin Immunol* 2016;■■■■:■■■■-■■■■.)

Key words: Sjögren's syndrome, autoimmunity, mass cytometry, cytometry by time-of-flight, immunophenotyping, patient stratification, biomarker discovery, therapeutic target

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In most autoimmune diseases (AID), there is an unmet therapeutic need because of a lack of understanding of pathologic mechanisms. Primary Sjögren's syndrome (pSS) belongs to this category. pSS is an autoimmune epithelitis with prevalence estimates ranging from 0.01% to 0.3% of the general population.¹ pSS is characterized by a lymphocytic infiltration of the lacrimal and salivary glands, leading to xerophthalmia and xerostomia. Apart from dryness, one third of patients have systemic involvement that can include the dermatologic, renal, pulmonary, or

Abbreviations used

AECG:	American European Consensus criteria
AID:	Autoimmune disease
Bm:	Memory B
CyTOF:	Cytometry by time-of-flight
DMEM:	Dulbecco modified Eagle medium
ESSDAI:	EULAR Sjögren's syndrome disease activity score
FS:	Focus score
IHC:	Immunohistochemistry
LASSO:	Least Absolute Shrinkage and Selection operator
LSG:	Labial salivary gland
MXA:	Myxovirus resistance gene A
NK:	Natural killer
pDC:	Plasmacytoid dendritic cell
pSS:	Primary Sjögren's syndrome
RF:	Rheumatoid factor
SSA:	Sjögren's syndrome A antigen
SSB:	Sjögren's syndrome B antigen

neurologic systems. Development of lymphoma is an additional severe complication that affects around 5% of patients.²

Cellular characterization of the immune infiltrate in exocrine glands from patients with pSS has relied thus far on nonquantitative immunohistochemistry (IHC) methods with low dimensionality, resulting in only partial understanding of disease pathogenesis (reviewed by Nocturne and Mariette³). IHC studies identified persistent inflammatory foci composed of T, B, and natural killer (NK) lymphocytes; ectopic germinal centers in 10% to 30% of patients; and progressive acinar epithelial cell atrophy and fibrosis.^{4,5} Similarly, the characterization of cellular variations in the blood of these patients has been focused on one or a few cell types by using low-dimensionality flow cytometry analyses. No study has reported phenotypic analyses on the exocrine glands and blood from the same patients. Because the target tissues in patients with pSS, as well as in those with other AIDs, are not easily amenable to repeated sampling, such a correlative study would help identify relevant blood biomarkers to monitor clinical interventions.

In 2011, Bendall et al⁶ described a new cytometric technology based on the detection of metal-conjugated antibodies by using cytometry by time-of-flight (CyTOF). Because antibodies used for mass cytometry are conjugated to isotopically pure rare earth elements, mass cytometry is not limited by the spectral overlap that limits flow cytometry, allowing for detection of up to 40 protein markers simultaneously with single-cell resolution. After antibody staining, cells are nebulized and sent to an argon plasma, ionizing the metal tags, which are then analyzed with a time-of-flight mass spectrometer. Since this first report, this approach has been used to deconvolute complex mixtures of cells and signaling events.⁶⁻¹¹ As reviewed by Cheung and Utz,¹² use of this approach for clinical research and drug development is promising because its high multiplex capabilities could allow resolution of the complexity and heterogeneity of human blood and tissues. Despite these prospects and considerable enthusiasm for this new approach,¹³ only a limited number of clinical applications have been reported.^{8,14-18} In addition, no study has used mass cytometry for clinical immunophenotyping of small samples, even though it is a robust approach to profile as few as 1000 cells.^{17,19}

Here we report a cross-sectional immunophenotyping study on a well-stratified cohort of patients with pSS, a prototypical example of both systemic and organ-specific AID. We used mass cytometry to immunophenotype peripheral blood from 49 patients with pSS and 45 control subjects and matched labial salivary gland (LSG) biopsy specimens from a third of these same subjects. We defined a blood 6-cell disease signature that clustered patients into groups with different disease activity, likely representing different forms or stages of the disease. In the LSGs, the target tissue of the disease, we identified new cellular phenotypes that correlated with some of the cellular variations observed in blood, pointing to new pathogenic mechanisms and candidate biomarkers.

METHODS**Study participants**

All patients with pSS (n = 49) met the American European Consensus criteria (AECG) diagnostic criteria.²⁰ Patients were referred to the Department of Rheumatology of Hôpitaux Universitaires Paris-Sud (France) between December 2013 and September 2014. Control subjects (n = 45) were either healthy volunteers or patients with sicca syndrome with a negative diagnostic result for pSS. Clinical evaluation confirmed that the latter had neither autoantibodies nor a positive biopsy result. The study was approved by the local research ethics committee, and informed written consent was obtained from all patients and control subjects. Additional information on the study design is available in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Preparation of single-cell suspensions

PBMCs were isolated by Ficoll gradient from whole blood within 4 hours of blood draw (heparin tubes). LSG biopsy specimens were obtained at the time of diagnosis. One to 2 glands were collected in Dulbecco modified Eagle medium (DMEM) supplemented with antibiotics, sodium pyruvate, nonessential amino acids, and glutamine. The remaining glands (2-4 glands) were formalin fixed for histologic analysis. Salivary glands were processed immediately after surgical collection by using a protocol that was optimized on mouse salivary glands to maximize cell recovery while minimizing effects on the expression of surface markers. Glands were first minced with scissors and enzymatically digested at 37°C under rotating agitation (100 rpm) in DMEM containing 232 U/mL collagenase II (Worthington Biochemical, Lakewood, NJ) and 8 U/mL DNase I (Sigma, St Louis, Mo). After 2 washes in calcium- and magnesium-free PBS containing 1 mmol/L EDTA, cellular aggregates were resuspended in 0.5 mL of TrypLE Express Enzyme (Gibco, Carlsbad, Calif) and incubated for 2 minutes at 37°C. Enzyme inactivation was achieved by means of dilution with 4 mL of DMEM and immediately followed by gentle pipetting with a P1000 to facilitate cell dissociation. The resulting cell suspension was washed twice with medium supplemented with 0.8 U/mL DNase I. Single-cell suspensions from blood and LSGs were resuspended at 10×10^6 /mL in PBS and fixed in 2% methanol-free formaldehyde (Electron Microscopy Sciences, Hatfield, Pa) for 20 minutes at room temperature. After 2 washes in PBS-0.5% BSA-0.02% sodium azide, cells were resuspended in PBS, and aliquots containing a maximum of 2×10^6 cells each were quickly frozen on dry ice and stored at -80°C until shipment to Biogen (Cambridge, Mass) for mass cytometry analysis.

Mass cytometry

Paraformaldehyde-fixed and frozen cell suspensions were thawed on ice. Samples were stained and prepared for CyTOF analysis, as previously described,¹⁰ by using an optimized cocktail of 34 metal-conjugated antibodies designed to identify 40 major and minor human blood cell subsets (see [Table E1](#) in this article's Online Repository at www.jacionline.org). After acquisition on a CyTOF II (Fluidigm, South San Francisco, Calif), data were normalized to internal bead standards.

IHC, immunofluorescence, and digital image analysis

IHC and immunofluorescence procedures were performed on the Ventana Ultra (Roche, Mannheim, Germany), unless otherwise indicated, by using a panel of optimized antibodies (see Table E2 in this article's Online Repository at www.jacionline.org). Details about digital image analysis and conditions for IHC and immunofluorescence procedures are available in the Methods section in this article's Online Repository.

Computational and statistical analyses

Automated cell clustering was performed with the viSNE algorithm available at www.cytobank.org.²¹ viSNE was used to define an optimal gating hierarchy on blood cell populations and to explore biopsy cell composition in an unbiased manner. Detailed methods for computational and statistical analyses of mass cytometry data are provided in the Methods section in this article's Online Repository.

RESULTS

Identification of a cellular signature in peripheral blood from patients with pSS

The correlation between flow and mass cytometry was very good in PBMCs from 9 healthy donors (average $R^2 = 0.92 \pm 0.09$), which confirmed the validity of mass cytometry for clinical immunophenotyping (see Fig E1 in this article's Online Repository at www.jacionline.org). Characteristics of the 49 patients with pSS and 45 control subjects are summarized in Table E3 in this article's Online Repository at www.jacionline.org. Using the viSNE algorithm,²¹ we identified 40 peripheral blood cell types and subsets and delineated an optimal gating strategy to manually define these populations using bidimensional plots (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Both abundant and rare cell types, such as plasmacytoid dendritic cells (pDCs), plasmablasts, or CD56^{bright} NK cells, were detected and quantified in all 94 PBMC samples (see Fig E2, B).

Principal component analysis revealed a right-skewed distribution of pSS samples along the first component of variation (PC1), indicating that the set of cellular variations contributing to PC1 distinguished patients with pSS from control subjects (Fig 1, A). Among the 40 cell subsets quantified, 6 populations were identified as differential between subjects with and those without pSS and contributed to this differential distribution along PC1: CD4⁺ T and memory B (Bm) lymphocytes, pDCs, activated HLA-DR⁺ CD4 and CD8 T cells, and plasmablasts.

We next analyzed separately patients with and without anti-Sjögren's syndrome A antigen (SSA) autoantibodies as autoantibody status was previously associated with differences in pathophysiology and the frequency of systemic manifestations of the disease.²²⁻²⁴ Patients with pSS had decreased lymphocyte counts in circulating blood in agreement with a recent report (Fig 1, B).⁵ This was mostly the result of a selective decrease in numbers of CD4⁺ T lymphocytes (Fig 1, C). NK cell counts were slightly but significantly reduced in patients with anti-SSA⁺ pSS. Naive, memory, and effector subsets of CD4⁺ T lymphocytes were all significantly decreased in patients with anti-SSA⁺ pSS, but the naive subset was the most affected (see Fig E3 in this article's Online Repository at www.jacionline.org). These features were not caused by background therapy because they were also observed in untreated patients (see Fig E4 in this article's Online Repository at www.jacionline.org). Moreover,

in patients with anti-SSA⁺ pSS, CD4⁺ and CD8⁺ T cells showed clear signs of activation, with a marked upregulation of HLA-DR (Fig 1, D). Although overall B-cell numbers were not significantly changed (Fig 1, C), there was an imbalance between subsets, with a 1.6-fold decrease in the frequency and absolute number of memory B cells (Fig 1, E). This decrease was consistent with previous findings.^{25,26} In contrast, an increased absolute number of plasmablasts was selectively observed in patients with anti-SSA⁺ pSS (Fig 1, E). Finally, we found a decrease in the number of circulating pDCs, which was limited to patients with anti-SSA⁺ pSS (Fig 1, F) and confirmed an earlier study.²⁷

Based on Least Absolute Shrinkage and Selection operator (LASSO) analyses,²⁸ pDCs, CD4⁺ T cells, Bm cells, and HLA-DR⁺ CD4⁺ T cells were the most important cell subsets to predict pSS diagnosis, with an area under the receiver operating characteristic curve equal to 0.89 in the training data and 0.86 in the test data (see the Methods section in this article's Online Repository for definition of the 2 cohorts and Fig E5 in this article's Online Repository at www.jacionline.org). Four of the 6 main differential populations showed strong correlations between them (Fig 1, G): decreases in CD4⁺ T and Bm cell numbers were positively correlated ($P = 3 \times 10^{-12}$, $r = 0.7$), and both variations were negatively correlated with the activation of CD4⁺ ($P = 2 \times 10^{-9}$ and 8×10^{-5} and $r = -0.63$ and -0.44 , respectively) and CD8⁺ ($P = 5 \times 10^{-5}$ and 1×10^{-3} and $r = -0.45$ and -0.37 , respectively) T cells. In contrast, the decrease in pDC and increase in plasmablast numbers were only weakly (pDCs) or not (plasmablasts) correlated with the 4 other cell populations, thus indicating that these variations likely identified different subsets of patients.

A cellular signature that defines subsets of pSS patients and correlates with disease activity

To explore further the biological relevance of this 6-cell disease signature, we next restricted the analysis to patients with pSS. Activity of the disease (assessed by the EULAR Sjögren's syndrome disease activity score [ESSDAI]²⁹) was negatively correlated with CD4⁺ T-cell and Bm cell numbers but positively associated with the frequency of activated CD4⁺ and CD8⁺ T cells (Fig 2, A). Classical serologic markers of the disease (anti-SSA/Sjögren's syndrome B antigen [SSB] positivity, IgG level, and rheumatoid factor [RF]) were associated with some cellular subsets of this signature (Fig 2, A).

Unsupervised hierarchic clustering of patients with pSS using solely the previously defined signature grouped the patients into 5 main clusters (Fig 2, B). Interestingly, patients with moderate or high activity (defined as ESSDAI score ≥ 5) were all contained within clusters 4 and 5 (Fig 2, B), indicating that this signature not only segregated patients from control subjects but also patients with the most active forms of the disease from the ones with less active forms of the disease. Patients in clusters 4 and 5 had the largest decrease in the CD4⁺ T and Bm cell numbers (Fig 2, C) and the highest proportion of activated CD4⁺ and CD8⁺ T cells (Fig 2, D). However, they differed with respect to pDC and plasmablast numbers (Fig 2, C). In contrast to clusters 4 and 5, clusters 1 to 3 were all associated with low disease activity (Fig 2, E) but corresponded to different subsets of patients. For instance, cluster 3 only contained anti-SSA⁺ patients and showed the highest IgG levels and highest plasmablast numbers, whereas clusters 1 and

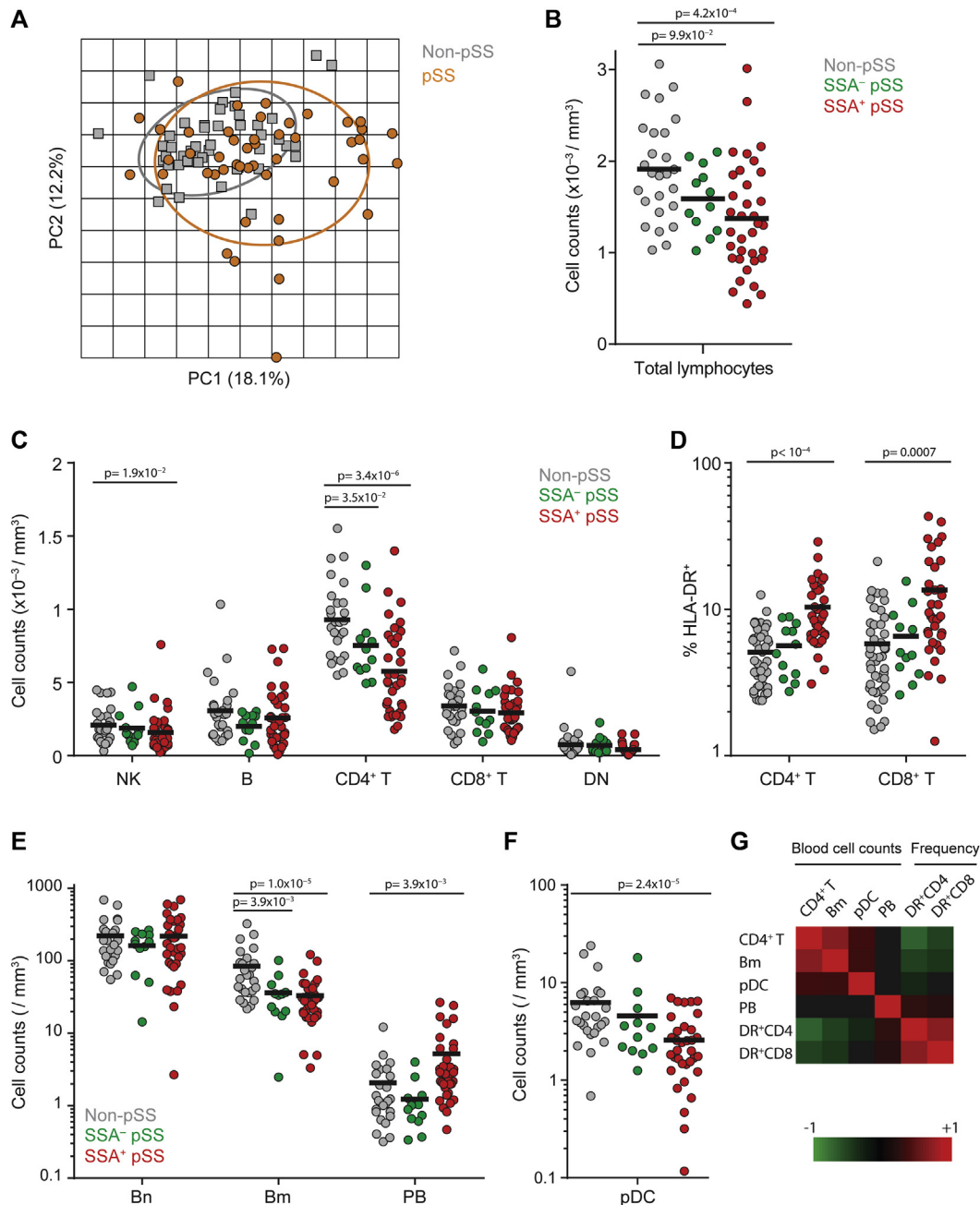


FIG 1. CyTOF immunophenotyping of peripheral blood from patients with pSS and control subjects. **A**, Principal component analysis using the frequencies of all 40 cell subsets detected by using CyTOF in patients without and with pSS (see key), with ellipses showing the overall distribution for each group. *PC1*, First component of variation; *PC2*, second component of variation. **B–F**, Absolute blood cell counts (Fig 1, B, C, E, and F) or relative frequencies (Fig 1, D) of the indicated cell types in each group of subjects (see key). Significant *P* values are indicated (Mann-Whitney test). **G**, Heat map of Spearman correlation coefficients between indicated cell types. Nonsignificant correlations are shown in black. *Bm*, Memory B; *Bn*, naive B; *PB*, plasmablasts.

2 identified subsets of patients with much more muted disease both at the cellular and biological levels (Fig 2, C–E). We next confirmed that the cellular signature was associated with disease activity by generating a composite *z* score reflecting the numbers of CD4⁺ T and Bm lymphocytes and frequencies of activated CD4⁺ and CD8⁺ T cells (see the Methods section in this article's Online Repository). Patients with a composite score greater than the threshold value contained all the patients

with moderate and high disease activity (ESSDAI ≥ 5 , 8/8 patients; Fig 2, F).

Deep immunophenotyping of LSG biopsy samples from patients with pSS

Because cellular variations in blood only indirectly inform disease pathogenesis, we developed a tissue dissociation protocol

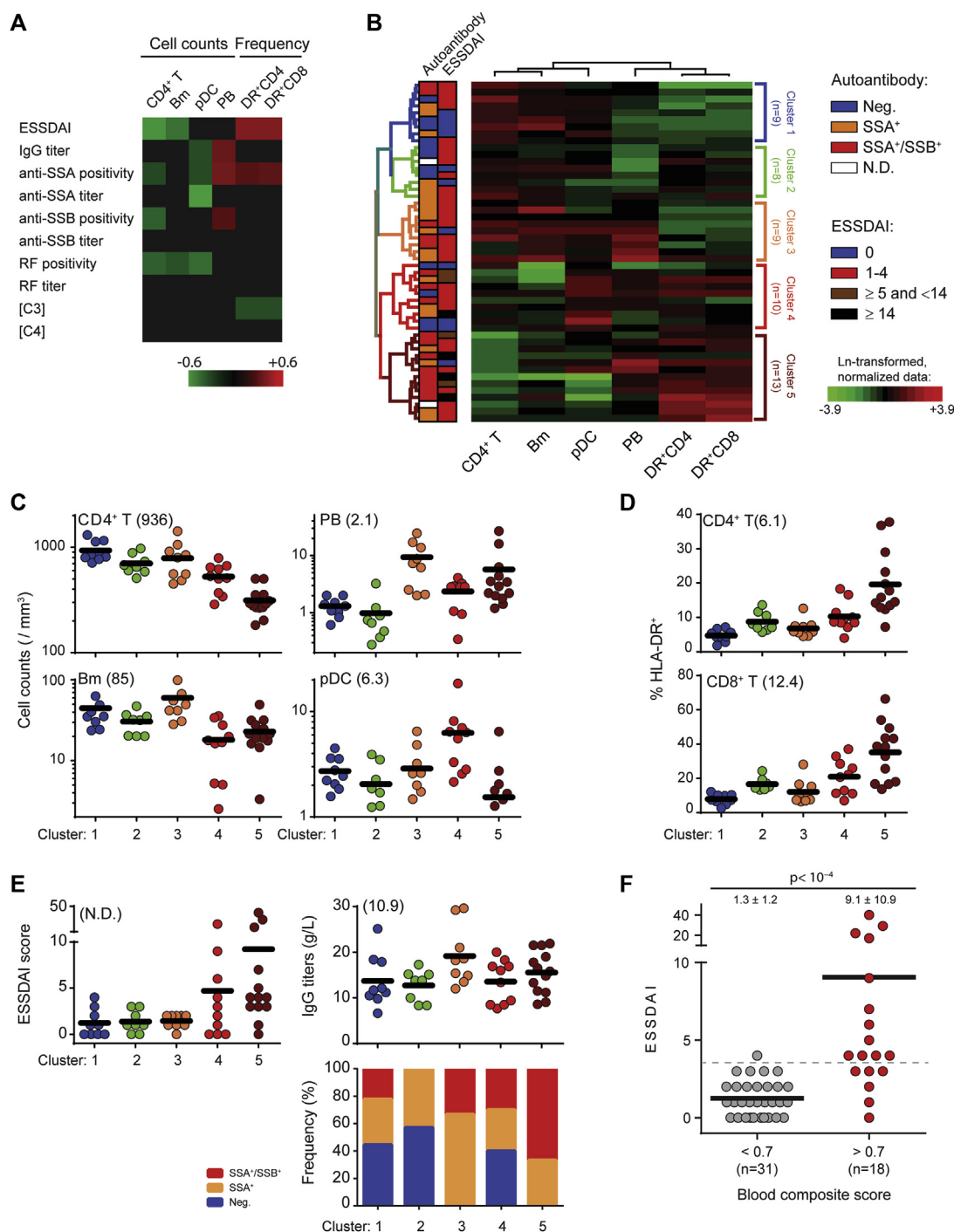


FIG 2. A disease signature stratifying patients with pSS according to disease activity. **A**, Heat map of Spearman correlation coefficients between blood cellular variations and clinical parameters. This analysis only includes patients with pSS. Nonsignificant correlations are shown in *black*. **B**, Dendrogram resulting from unsupervised hierarchical clustering of patients with pSS by using the 6 cell types from the pSS signature. Autoantibody status and ESSDAI scores are indicated (see key). *Bn*, Naive B; *N.D.*, not determined; *PB*, plasmablasts. **C-E**, Absolute blood cell counts (Fig 2, C) or relative frequency (Fig 2, D) of the indicated cell types and clinical variables (Fig 2, E) for each cluster of patients identified in Fig 1, B. The averaged value for patients without pSS is indicated in brackets (top left of each plot). **F**, ESSDAI scores for patients with a blood composite score of less than and greater than 0.7 (see the [Methods](#) section). *P* values were determined by using the Mann-Whitney test.

to prepare single-cell suspensions from biopsy specimens taken from LSGs, the target tissue in pSS. We isolated enough cells to perform CyTOF analysis on 29 subjects (average number of nucleated cells per patient, 3,248 [775–17,885]), 16 with pSS and 13 with sicca symptoms without any immunologic abnormalities (see Table E4 in this article's Online Repository at www.jacionline.org).

Clustering analyses with the viSNE algorithm were performed to identify cell subsets in an unbiased manner (Fig 3, A). Two main cell types were identified by anti-CD326 (epithelial cell adhesion molecule) and anti-CD45 antibodies: epithelial and hematopoietic cells. We normalized for subject-to-subject variations in the number and size of LSGs by calculating the number of cells detected for 10 epithelial cells for each hematopoietic subset. We found good agreement between the number of CD45⁺ cells identified by means of mass cytometry and the focus score (FS) measured on different glands from the same subject by using histology (Fig 3, B). viSNE analyses revealed that most of the CD45⁺ infiltrating cells in patients with pSS with a positive biopsy result (FS ≥ 1) were CD3⁺ T cells, which were also detected in subjects without pSS, although in much lower numbers (Fig 3, A and B). Most of these T cells had a CD45RO⁺ memory phenotype in both subjects with and those without pSS (data not shown).

The comparative analysis of biopsy samples from subjects with and without pSS revealed 3 main findings. First, even though both CD4⁺ and CD8⁺ T cells were detected in the glands of subjects with and without pSS, only CD8⁺, but not CD4⁺ T lymphocytes from patients with pSS showed a significant increase in HLA-DR (Fig 3, C). Second, the most abundant hematopoietic population after T cells in patients with pSS and positive biopsy results was a population of CD3⁺CD38⁺⁺CD27⁺ cells, which was only detected at very low levels in patients without pSS (Fig 3, A and B). Up to 40% to 50% of these cells were CD19⁺ (Fig 3, A), indicating that a significant fraction of these cells were fully differentiated CD19⁺ plasma cells. Accordingly, blood and tissue CD38⁺⁺CD27⁺ cells were both negative for CD20, and only tissular, but not blood, CD38⁺⁺CD27⁺ cells did not express HLA-DR, a hallmark of differentiated plasma cells (see Fig E6 in this article's Online Repository at www.jacionline.org).³⁰ The third key observation was a prominent upregulation of HLA-DR on salivary epithelial cells seen in 60% of patients with pSS with positive biopsy results (Fig 3, A and D, and see Fig E7 in this article's Online Repository at www.jacionline.org) but absent in all subjects without pSS. This observation confirmed previous IHC analyses of LSGs from patients with pSS and cultured salivary gland epithelial cell lines.^{31–33} An increase in the levels of expression of HLA-A,B,C was also observed in epithelium from patients with pSS (Fig 3, D).

Validation and exploration of anatomic relationships at the tissue level

We next validated CyTOF findings and explored anatomic distribution by using quantitative IHC and immunofluorescence methods in paired formalin-fixed LSGs from 15 (10 patients with pSS [5 with FS < 1 and 5 with FS ≥ 1] and 5 subjects without pSS) of the 29 subjects we studied using CyTOF. Digital image analysis showed that the CD45⁺ area correlated well with FS ($P = .0002$, $R^2 = 0.7$; Fig 4, A and B). As identified in biopsy specimens evaluated by using CyTOF, patients with FS of 1 or greater had

large numbers of infiltrating CD8⁺ T cells within central inflammatory foci, regions that were also immunopositive with HLA-DR (Fig 4, C). In a small subset of individual CD8⁺ T cells, there was concentric colocalization of CD8 and HLA-DR signal (Fig 4, D).

pDCs, which were decreased in the blood of patients with pSS (Fig 1, F), were not detectable in biopsy specimens by using CyTOF. Nevertheless, by using IHC, infrequent large round CD303⁺ cells (pDCs) were found within inflammatory foci in biopsy specimens from patients with pSS, which is in agreement with a previous study (Fig 4, E).³⁴ Percentage area immunopositive with CD303 in patients with pSS was not significantly different than in subjects without pSS (data not shown). Using quantitative IHC, we found 30 to 50 times fewer pDCs than CD8⁺ T cells on average, likely explaining their lack of detection by using CyTOF. There was no correlation between numbers of blood pDCs detected by using CyTOF and glandular pDCs detected by using IHC in the same patients. The interferon-induced myxovirus resistance gene A (MXA) protein was rarely detected but was present in the ductal epithelial and inflammatory cells in 2 of the 3 patients with the highest CD303⁺ area (Fig 4, E).

In agreement with CyTOF findings, there was a significant increase in HLA-DR immunopositivity in the epithelium from patients with pSS. Relative to the glandular periphery, regions adjacent to inflammatory foci were mildly enriched for HLA-DR–immunopositive signal (Fig 5, A and B). Morphologic analysis of hematoxylin and eosin–stained sections revealed large numbers of well-differentiated plasma cells in patients with pSS in the interstitium surrounding acinar tubules (Fig 5, C), which confirmed results obtained with CD38 and CD27 markers by using CyTOF (Fig 3). Quantification of CD138⁺ round cells further validated that the number of plasma cells was significantly increased in patients with pSS (average fold increase = 3.9 for patients with FS ≥ 1; Fig 5, D–F). Only a small fraction of CD138 signal colocalized with HLA-DR ($0.22\% \pm 0.18\%$; Fig 5, G), which is consistent with a mature long-lived plasma cell phenotype.

Cellular variations in LSGs correlate with clinical and biological markers of the disease

The overall number of immune infiltrating cells (CD45⁺) detected by using CyTOF in LSGs, specifically T and B cells, was positively correlated with pSS diagnosis according to AECG ($P = .009$, $r = 0.5$), the histologic assessment of glandular infiltration using FS ($P = .0005$, $r = 0.55$), and the frequency of epithelial cells with upregulated HLA-DR ($P = .0001$, $r = 0.67$; Fig 6, A and data not shown). In contrast, blood lymphocyte counts were negatively correlated with both numbers of glandular CD45⁺ cells ($P = .03$, $r = -0.42$) and frequencies of HLA-DR⁺ epithelial cells in target tissue ($P = .018$, $r = -0.45$; Fig 6, A), suggesting that lymphopenia might be caused by cell migration into the glandular tissue.

As conveyed in Fig 6, A, the number of immune infiltrating cells was also positively associated with the ESSDAI score ($P = .016$, $r = 0.5$), supporting a link between local glandular inflammation and systemic manifestations of the disease. Specifically, the 2 immunologic features that showed the most significant correlations with the ESSDAI score were (1) glandular B-cell numbers ($P = .006$, $r = 0.52$), confirming the pathogenic role of B cells in this disease, and (2) the frequency of activated CD8⁺ T cells ($P = .007$, $r = 0.51$), identifying a new cellular player in disease

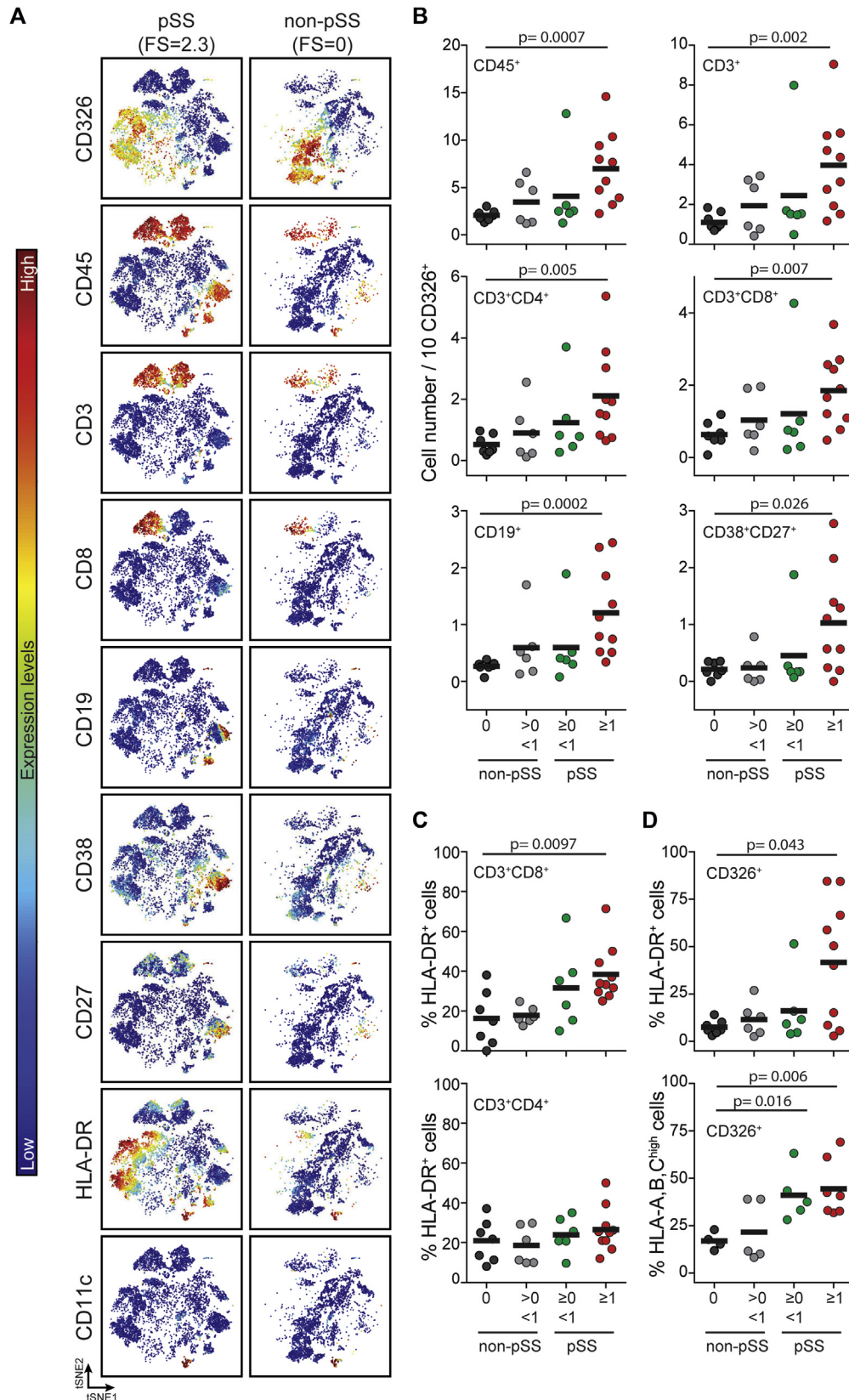


FIG 3. CyTOF immunophenotyping of lip biopsy specimens from patients with pSS and control subjects. **A**, viSNE maps of nucleated events detected by using CyTOF in LSG cell suspensions from representative patients without pSS and with pSS. Each dot represents a cell. Colors show levels of expression of indicated markers. **B**, Quantification of cell types identified in Fig 3, A. Patients without and those with pSS are grouped according to biopsy FS. **C**, Frequency of HLA-DR⁺ cells among T8 (top) and T4 cells (bottom). **D**, Frequency of HLA-DR⁺ (top) and HLA-A,B,C^{high} cells (bottom) among CD326⁺ epithelial cells. Significant *P* values are indicated (Mann-Whitney test).

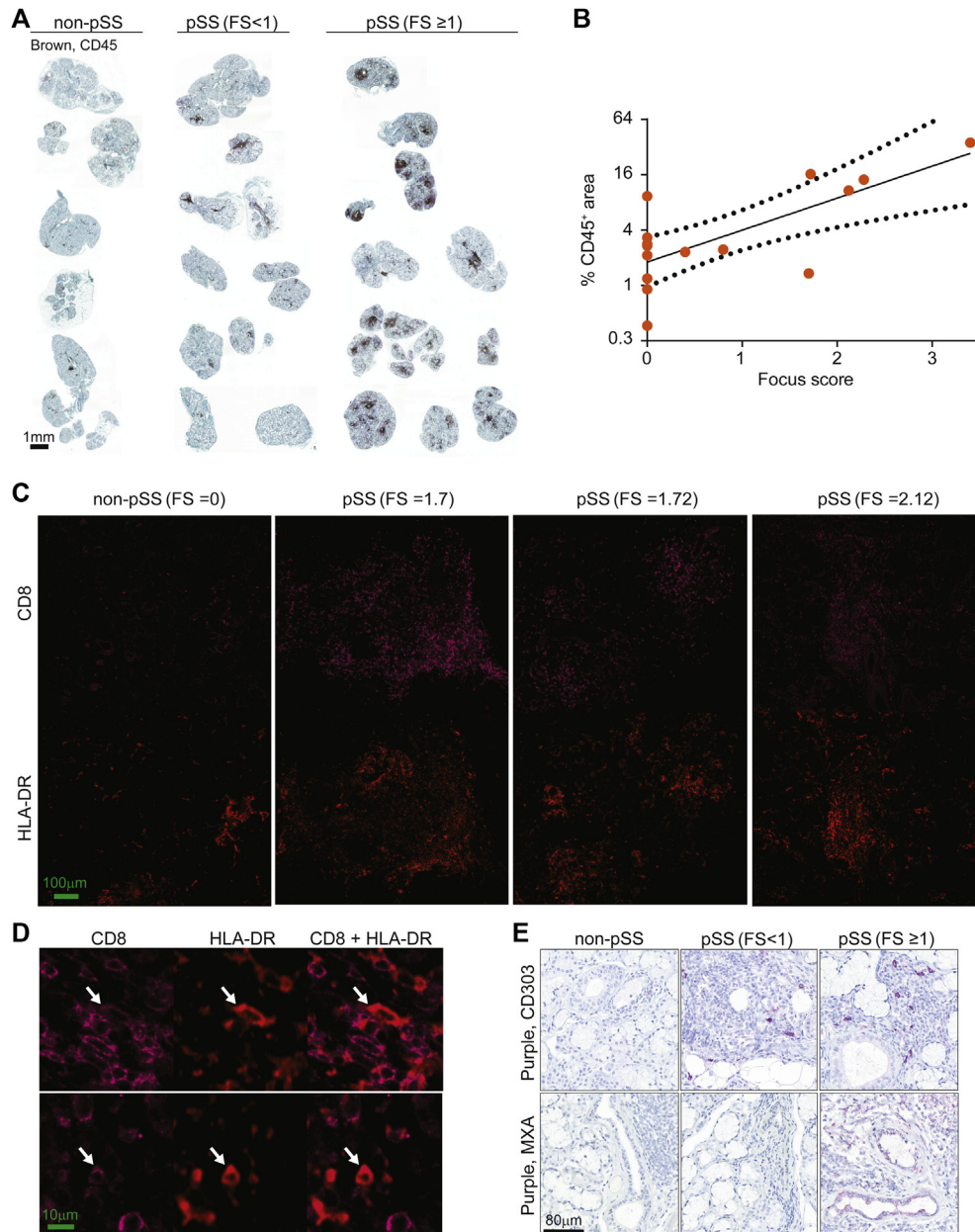


FIG 4. Anatomic relationships within foci of immune infiltration in biopsy specimens from 10 patients with pSS and 5 control subjects. **A**, Lip biopsy specimens segmented by FS and reacted against anti-CD45 (brown), anti-HLA-DR (purple) with light trichrome and green counterstain. **B**, Digital analysis and quantification of Fig 4, A, relative to total glandular area. **C**, Representative photomicrographs of CD8 (pink) and HLA-DR (red) signals in focal lymphocytic aggregates from indicated subjects. **D**, Higher magnification of Fig 4, C. **E**, Representative photomicrographs of CD303 (purple, top) and MXA (purple, bottom) signals.

pathogenesis (Fig 6, A). Activated CD8⁺ T-cell numbers also correlated with the presence of anti-SSA/SSB antibodies ($P = .002$, $r = 0.57$) and RF serology ($P = .027$, $r = 0.43$). Interestingly, glandular plasma cell numbers did not correlate with disease activity but with serologic markers: serum IgG level ($P = .047$, $r = 0.39$) and positive RF ($P = .029$, $r = 0.42$).

Identification of blood biomarkers of glandular inflammation

In total, paired biopsy and blood immunophenotyping data were available for 27 (15 patients with pSS and 12 subjects

without pSS) of the 29 subjects with glands studied by using CyTOF, allowing us to identify blood proxy markers of glandular inflammation. Two blocks of correlation emerged from this analysis (Fig 6, B): (1) a negative correlation between blood CD4⁺ T- and Bm cell numbers and glandular immune infiltration and activation and (2) a positive correlation between the blood frequency of activated CD4⁺ and CD8⁺ T cells and multiple cellular parameters in the biopsy, among which was the degree of activation of the epithelium. Thus the 4 cellular parameters from the blood pSS signature that were associated with the ESSDAI score and most differential between blood clusters 1 to

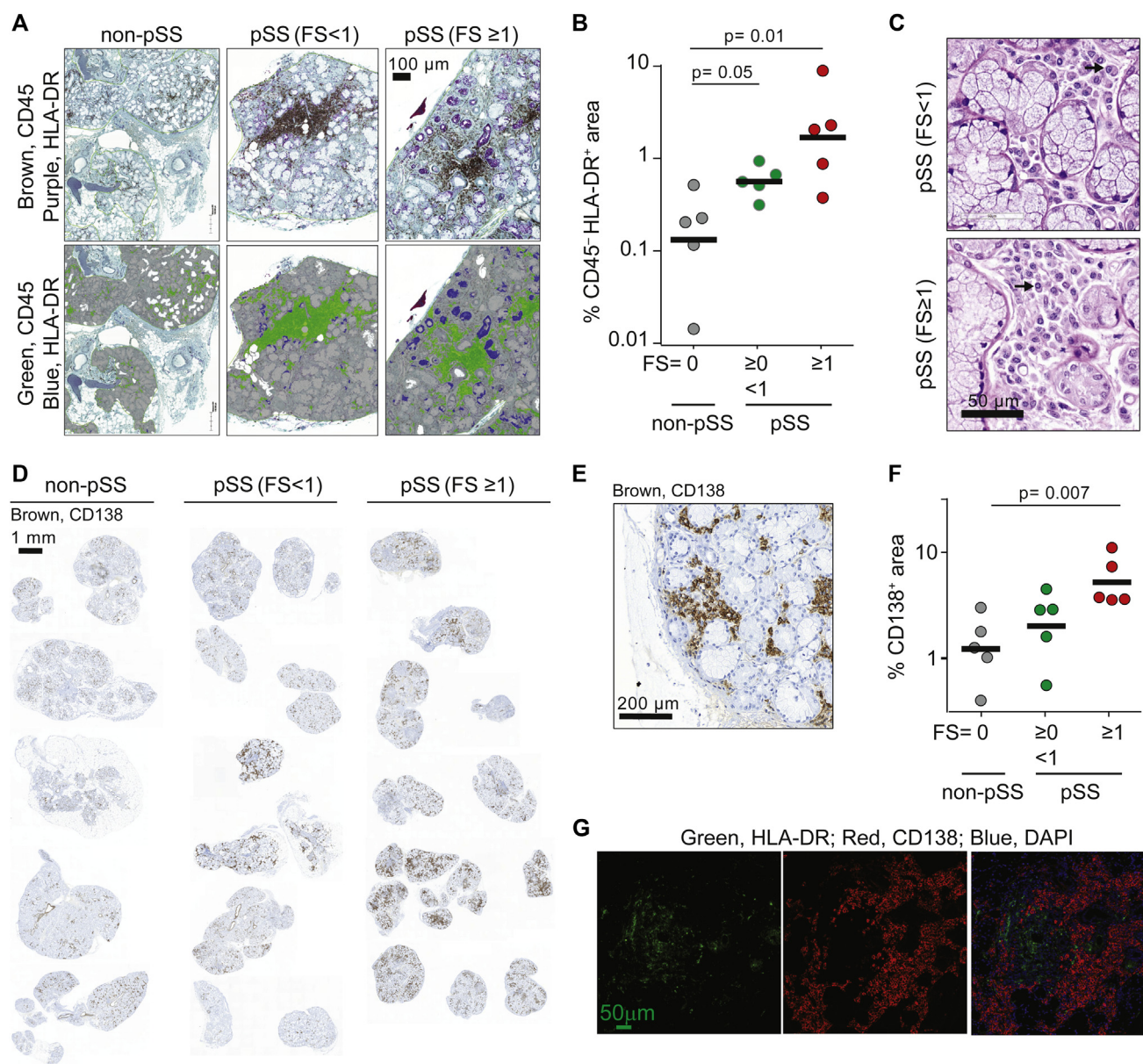


FIG 5. Anatomic relationships outside of immune foci in biopsy specimens from patients with pSS. **A**, Digital quantification of HLA-DR⁺ epithelium (purple, top row; blue, bottom row). Interlobular fibrous connective tissue and CD45⁺ cells (gray and green areas, respectively, bottom row) were excluded. **B**, Quantification of Fig 5, A, relative to total lobular area. **C**, Hematoxylin and eosin–stained sections from 2 patients showing well-differentiated plasma cells within the interstitium (arrows). **D**, Lip biopsy specimens segmented by FS and reacted against anti-CD138 (brown). **E**, Higher magnification of Fig 5, D, from a patient with an FS of greater than 1. **F**, Quantification of Fig 5, D, relative to total tissue area. Immunoreactive epithelium was excluded. **G**, Immunofluorescence detection of indicated markers in a representative gland from a patient with pSS. DAPI, 4'-6-Diamidino-2-phenylindole dihydrochloride.

3 and clusters 4 and 5 (Fig 2) also showed significant correlations with immunologic activity within the glandular tissue. Accordingly, when we used the pSS blood 6-cell signature to stratify patients into the corresponding 2 groups, we observed that patients from clusters 4 and 5 had more severe immune infiltration in LSG tissues (Fig 6, C). Altogether, these results indicated that the blood signature stratified patients into subsets with not only distinct disease activity but also differential immunologic activity within the target tissue.

DISCUSSION

This study is the first large-scale clinical application of mass cytometry to stratify a patient population with a chronic disease. In addition, this is also the first study that performs immunophenotyping of paired blood and biopsy samples to correlate cellular changes in the blood and target tissue of the disease. The strength of this high-dimensional and unbiased immunophenotyping approach relies on its ability to identify new cellular variations and, importantly, to enable the simultaneous measurement of

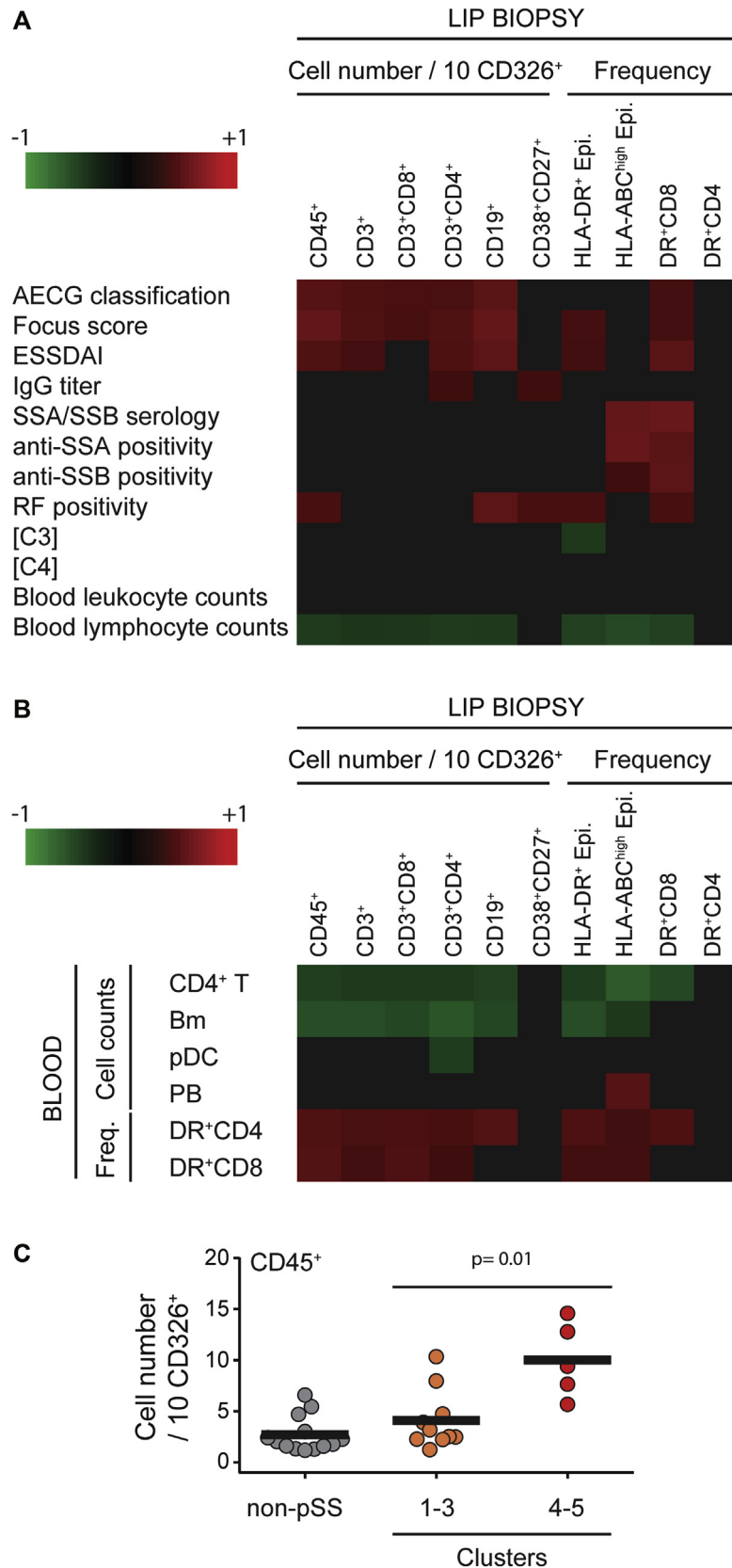


FIG 6. Correlation between cellular variations in LSGs, clinical parameters, and the pSS blood signature. **A** and **B**, Heat map of Spearman correlation coefficients between cellular variations observed in lip biopsy specimens and clinical parameters (Fig 6, **A**) and blood cellular variations (Fig 6, **B**). Nonsignificant correlations are shown in black. *PB*, Plasmablasts. **C**, Number of CD45⁺ infiltrating cells in biopsy specimens from subjects without and those with pSS belonging to indicated clusters (see Fig 2, **B**, for cluster definition).

these variations, as well as their correlation with each other and with clinical and biological markers of the disease. This novel approach allowed us to identify 6 unique cellular phenotypes in the blood of patients with pSS, which altogether defined a cellular signature characteristic of the disease state. A linear combination of 4 of these cellular variations was sufficient to predict pSS diagnosis with good accuracy. Future studies will establish whether these cellular variations are specific to pSS or shared with other chronic AIDs.

In addition to discriminating patients with pSS from subjects without pSS, this signature also stratified patients into groups with distinct clinical and biological features (Fig 7). Clusters 4 and 5, which included all patients with moderate and high disease activity, shared some cellular features in the blood ($CD4^+$ T and Bm lymphopenia and higher frequencies of activated $CD4^+$ and $CD8^+$ T lymphocytes), pointing to commonalities in disease pathogenesis. In contrast, these 2 clusters had very different representation of pDCs and plasmablasts, indicating that both shared and distinct pathogenic pathways are at play in different patient subsets. Clusters 4 and 5 also had the most severe immune infiltration in the glandular tissue (Fig 7), indicating that the blood pSS signature could be used as a good biomarker and dynamic proxy of immunologic activity in the target tissue in clinical trial settings in which LSG biopsy sampling is not easily repeated.

The fact that some of these cellular variations were already reported in the literature demonstrates the validity of this technical approach for patient immunophenotyping. This is the case in particular for memory B cells, whose decrease is one of the best characterized cellular variation in the blood of pSS patients and was recently shown to have diagnostic value.^{25,26} Our study found additional dysregulation of B-cell subsets. Increased numbers of plasmablasts and plasma cells were identified in the peripheral blood and LSGs, respectively, of patients with pSS. In both cases we observed correlations between the number of these antibody-producing cells and serum IgG levels and autoantibody status. In the target tissue from pSS patients, 40 to 50% of B cells, located throughout the peripheral interstitium of glandular lobules, had a $CD38^{++}CD27^{+}CD20^{-}HLA-DR^{-}$ phenotype, indicating that a large number of the exocrine tissue-resident B cells in patients with pSS are fully differentiated plasma cells. These results confirm previous observations showing that resident plasma cells in LSGs are $Ki67^{-}$ and thus long-lived plasma cells.³⁵ Note also that a significant fraction of the $CD38^{+}CD27^{+}$ cells were $CD19^{-}$, a phenotype recently associated with long-lived maintenance of humoral immunity in human subjects.³⁶ Altogether, these observations support the idea that short-lived plasmablasts might recirculate from the LSGs, whereas long-lived plasma cells differentiate and accumulate within the exocrine tissue. The persistence of $CD20^{-}$ plasma cells could explain the only partial efficacy of rituximab and its absence of effects on anti-SSA/SSB titers. Identifying changes in the epithelium and stroma that convert the exocrine glands into a plasma cell niche will be of interest. In addition, plasma cells themselves or factors responsible for the trafficking of plasmablasts and plasma cells could also be potential therapeutic targets.

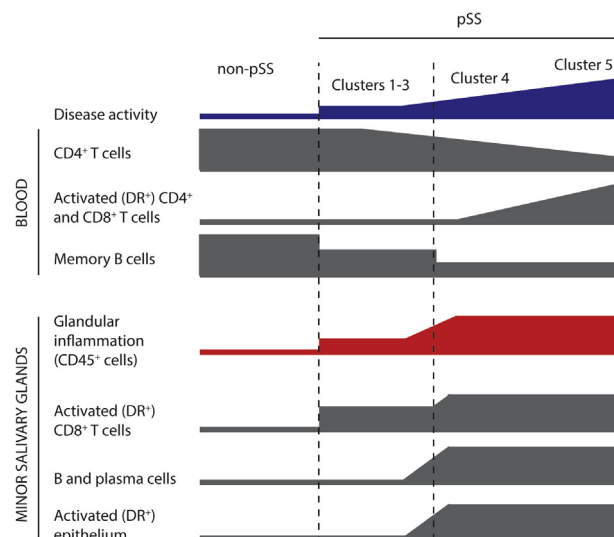


FIG 7. Model summarizing relationships between the key cell types from the blood pSS signature, disease activity, and glandular inflammation. Absolute numbers of $CD4^+$ T and Bm lymphocytes and frequencies of activated $CD4^+$ and $CD8^+$ T cells in the blood segregate patients into clusters with distinct disease activity (blue) and glandular inflammation (red). Major findings in LSGs from corresponding patient clusters are also depicted.

Lymphopenia was reported in some patients,⁵ even though its cause and biological significance remain unknown. The current study provides some clues on the origins of lymphopenia by attributing it to a selective reduction in all subsets of $CD4^+$ T cells and identifying strong correlations between blood $CD4^+$ T-cell numbers and (1) the number of lymphocytes within the target tissue, (2) the upregulation of HLA-DR in the glandular epithelium, and (3) disease activity. Even though these observations do not formally demonstrate that the migration of peripheral blood $CD4^+$ T cells to the exocrine glands is the cause of lymphopenia, they provide supporting evidence in favor of this model. Lymphopenia was previously associated with the occurrence of lymphoma.³⁷ The recent demonstration of the association of disease activity with an increased risk of subsequent lymphoma³⁸ supports the fact that lymphopenia is a disease activity marker. Likewise, our observation that $CD4$ T-cell lymphopenia was one of the key defining cellular factors of the 2 subsets of patients with most active disease (clusters 4 and 5) is in agreement with this finding. Future longitudinal studies should establish whether this very simple biomarker can be used as a dynamic measure of disease activity, response to treatment, or both.

The increase in activated T-cells in both the blood and exocrine glands from patients with pSS is another novel finding. In the blood both $CD4^+$ and $CD8^+$ T cells showed signs of activation, whereas surprisingly, only $CD8^+$ T cells showed upregulation of HLA-DR within the target tissue. HLA-DR expression on glandular $CD8^+$ T lymphocytes was strongly associated with many clinical features, including the ESSDAI score. These results point to a pathogenic role of $CD8^+$ T lymphocytes, which has been much less emphasized than the role of $CD4^+$ T cells. Because type 2 interferon is dominant in the target tissue,³⁹

CD8⁺ T lymphocytes could not only be one of the glandular cells secreting IFN γ but also a key cellular player in mediating tissue destruction. The exploration of the function of CD8⁺ T cells in patients with pSS is thus warranted.

For the first time, this study also confirms in an *ex vivo* analysis that LSG epithelial cells from patients with pSS upregulate HLA-DR and HLA-A,B,C, changes that were initially reported in cultured epithelial cells and on fixed tissues by using IHC (reviewed by Manoussakis and Kapsogeorgou⁴⁰). We extended this observation by demonstrating that these changes in the physiology of the epithelium tightly correlated with (1) the number of infiltrating cells within the gland, (2) CD4 T-cell lymphopenia, (3) peripheral blood T-cell activation, (4) autoantibody production, and (5) the ESSDAI score. Even though these correlations support a role of the epithelium in pathogenesis, it remains to be established that these changes are associated with functional antigen presentation capabilities. Identifying the broader molecular modifications taking place in the epithelium of patients with pSS will be of importance.

On the innate immunity side, the decrease in pDC counts in the blood of seropositive patients confirmed one earlier report.²⁷ However, it is difficult to reconcile with the fact that (1) the interferon signature is known to be mainly caused by pDCs and (2) the interferon signature was detected in both the salivary glands and blood of patients with pSS.^{23,39} Therefore it remains unclear at present how a selective decrease in pDC numbers in the blood causes an increase in the interferon signature. pDCs have been detected in the LSGs of patients with pSS, although in very low numbers.³⁴ In agreement with this study, we only detected low numbers of pDCs in biopsy specimens from patients with pSS, and their numbers did not correlate with pDC numbers in blood. The observed decrease in the number of pDCs in the blood of patients with pSS might thus result from their activation by immune complexes, interferon production, and induction of a negative feedback loop on pDC homeostasis rather than from their migration in the target tissue. Such a mechanism leading to type 1 interferon-induced pDC apoptosis was reported to occur during viral infections.⁴¹

Clustering patients using the blood disease signature indicates that each of these cell types can either act at different stages of disease or in different subsets of patients or forms of pSS (Fig 7). Future studies will focus on their interplay and causal roles in disease onset and activity. Some of these identified cell subsets might also represent new cellular targets for future drugs. This first application of mass cytometry to a well-stratified clinical cohort and small biopsy tissues clearly establishes the benefits of such an approach for identifying new biomarkers and new therapeutic targets in patients with AIDs.

We thank our Biogen colleagues, Nathalie Franchimont, Cristina Musselli, Jo Viney, Linda Burkly, Shauna Andersson, Bob Dunstan, Cherié Butts, and Tim Zheng, for discussion and feedback on the manuscript; the Biogen Biobank (Marcia Lara and Mark Cosentino) for their help with sample management; Jeffrey Yu for statistical programming support; Luke Jandreski, Stefan Hamann, and Daniel Sofia for developing IHC/immunofluorescence quantification algorithms; and Daniel Sofia for IHC/immunofluorescence image digitization.

Key messages

- Clinical application of mass cytometry to a well-stratified cohort of patients with Sjögren's syndrome defines a blood 6-cell disease signature stratifying patients into subsets with distinct disease activity and clinical features.
- CyTOF immunophenotyping of matched salivary gland biopsy specimens from the same patients reveals new cellular therapeutic targets for future clinical intervention and blood biomarkers that correlate with glandular inflammation.
- This study establishes the clinical benefits of mass cytometry and supports its future use in longitudinal and interventional clinical settings in this and other disease areas.

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METHODS

Study design

This observational case-control study was designed to identify cellular signatures in patients with pSS that correlate with disease activity and clinical features of the disease. Measurements included protein expression levels of 34 markers by using mass cytometry and of a more limited set of markers by using IHC and immunofluorescence selected based on mass cytometry results. Power analysis was not performed to calculate sample size because of the exploratory nature of this study. Samples were run in 2 sequential cohorts. The first cohort of samples was acquired on the CyTOF instrument once 50% of the samples were collected. Principal component analysis did not reveal batch effects, and independent analyses of each cohort revealed good agreement between cohorts. Therefore the analysis presented in the manuscript was performed on the combined cohorts. All blood samples were included in the analysis. Biopsy specimens were collected from a total of 36 subjects with suspicion of Sjögren's disease at the time of the diagnostic biopsy. We successfully isolated enough cells to perform CyTOF analysis from 32 subjects, among whom were 3 subjects with diseases other than Sjögren's syndrome, who were therefore excluded. We thus analyzed 29 subjects, 16 with pSS according to AECG and 13 with sicca symptoms without any immunologic abnormalities. Sample acquisition and analysis were conducted blindly up to the generation of cellular frequencies for all 40 cell subsets detected. Frequencies and cell numbers were then associated and correlated with clinical data tables by using a unique sample identifier and a custom-designed R-based tool, CytoShine (see computational analyses and statistical analyses of CyTOF data).

IHC, immunofluorescence, and digital image analysis

CD45/HLA-DR double IHC slides were counterstained with (1) Weigert iron hematoxylin (Rowley, Danvers, Mass), (2) Trichrome Stain LG (Sigma, St Louis, Mo), and then (3) 0.2% Fast Green (EMS, Hatfield, Pa). Anti-MXA mouse mAb IgG_{2a} (M143) was generously provided by Dr Gerog Kochs and used at a concentration of 0.3 µg/mL on the Ventana XT with the UltraMap Red anti-Ms detection kit (Roche). Whole slide images were acquired on 3D Histech Panoramic (IHC) and Olympus BX61VS (immunofluorescence) digital scanners (Olympus, Center Valley, Pa). Digital quantification of whole slide images was accomplished by custom designing for each experiment algorithms in Visiopharm (Hørsholm, Denmark) software. *P* values are from unpaired *t* tests or Mann-Whitney tests for parametric and nonparametric data sets, respectively.

Computational and statistical analyses

visNE was used to define an optimal gating hierarchy on blood cell populations and to explore biopsy cell composition in an unbiased manner. Cell subsets were next identified by means of manual gating with FlowJo software (TreeStar, Ashland, Ore), and the resulting cellular proportions were exported into Prism (GraphPad Software, La Jolla, Calif) and R software for statistical analyses.

Pairing of cellular variables with clinical data and systematic identification of significant cellular variations between subjects with and without pSS or

different clinical subgroups was performed by using a customized R-based tool (CytoShine). We next analyzed separately anti-SSA⁺ and anti-SSA[−] patients with pSS compared with control subjects because autoantibody status was previously associated with differences in pathophysiology, as well as in the frequency of systemic manifestations of the disease.^{E1-E3} CytoShine implements statistical models to identify cell populations correlated with clinical variables. Specifically, we modeled the number of cells within a given population using a negative binomial generalized linear model using the DESeq2 R package.^{E4} This model assumes that the error on the cell number counts follows a discrete negative binomial probability distribution. For a given clinical variable, we modeled its effect as a single factor and calculated the differential in cell population counts. For categorical variables with multiple levels, we computed significance for all pairwise combinations and reported the minimum adjusted *P* value together with fold changes. For continuous variables, we assumed a constant fold change was expected for each unit of the covariate.

P values from Mann-Whitney tests are displayed in all figures to compare distribution of cell subsets between different clinical groups. Correlation heat maps show the nonparametric Spearman rank correlation coefficient for *P* values of less than .05. Nonsignificant correlations are shown in black. Hierarchical clustering was performed on natural logarithm (ln)-transformed data. Data were shifted to a mean of 0 and SD of 1 before clustering by using Pearson dissimilarity distance and complete linkage. Predictive models of disease status used LASSO.^{E5} We first split all the samples into training data (the samples that were profiled in the first cohort) and test data (the samples that were profiled in the second cohort) and used 5-fold cross-validation to avoid overfitting issue. The R package glmnet was used to fit LASSO model.^{E6} The composite *z* score used to identify patients with high disease activity was generated by calculating individual *z* scores for CD4⁺ T (*Z*_{T4}) and Bm (*Z*_{Bm}) cell numbers and frequencies of activated CD4⁺ (*Z*_{T4DR+}) and CD8⁺ T cells (*Z*_{T8DR+}) and using the following formula:

$$Z = Z_{T4DR+} + Z_{T8DR+} - Z_{Bm} - Z_{T4}.$$

A high score reflected high frequencies of activated T cells and/or low numbers of Bm and/or low numbers of CD4⁺ T cells.

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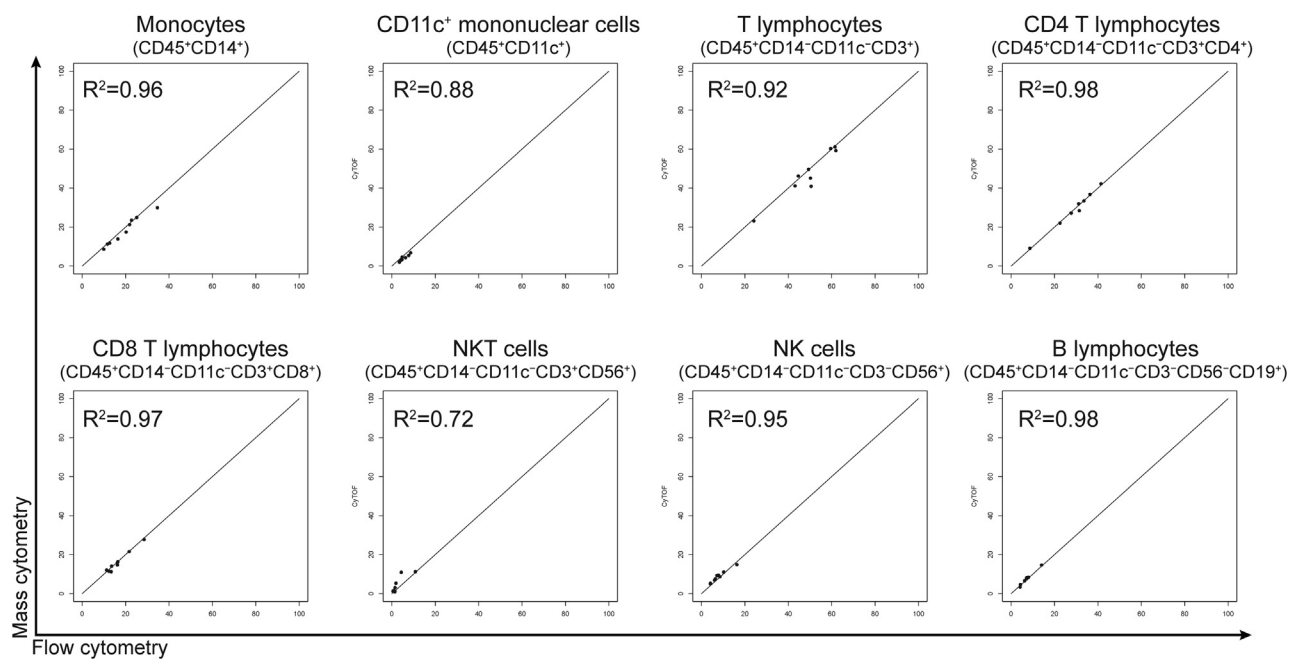


FIG E1. Validation of mass cytometry for immunophenotyping of clinical samples. PBMCs from 9 healthy donors were analyzed by means of flow and mass cytometry in parallel with 10 common antibody clones. Dot plots showing the frequency of 8 cell types measured by using flow (*x-axis*) and mass (*y-axis*) cytometry are shown. The gating hierarchy and linear regression coefficients are indicated.

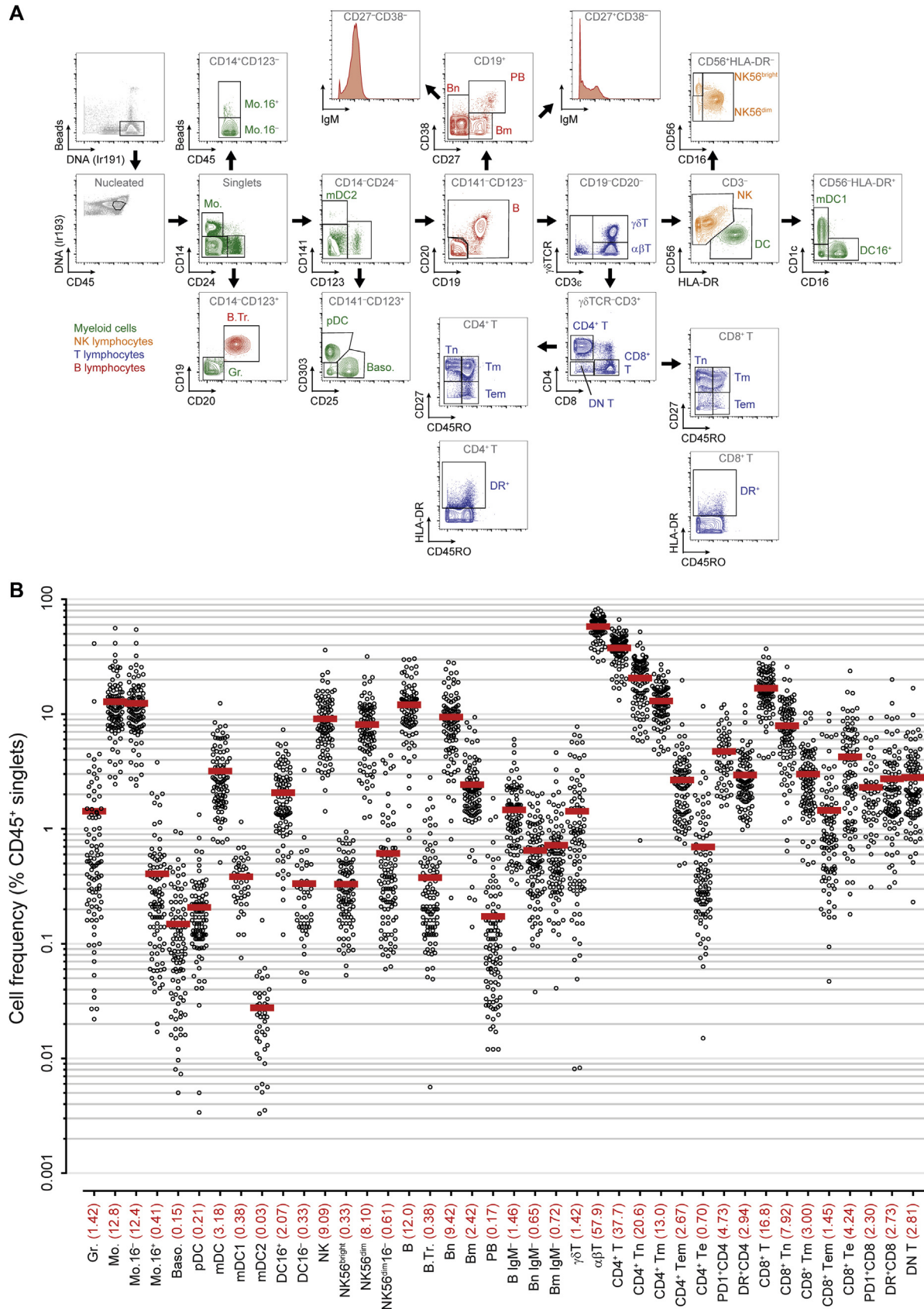


FIG E2. Identification and quantification of 40 cell types and subsets in peripheral blood by using CyTOF. **A**, Bidimensional gating strategy used to phenotype clinical samples. The parental population of each dot plot is indicated in gray. **B**, Frequency of each cell subset among CD45⁺ singlet mononuclear cells plotted for all subjects. Average frequency is indicated in parentheses and by a red line. *Baso.*, Basophils; *Bn*, naive B; *B.Tr.*, transitional B; *Gr.*, granulocytes; *mDC*, myeloid dendritic cells; *Mo.*, monocytes.

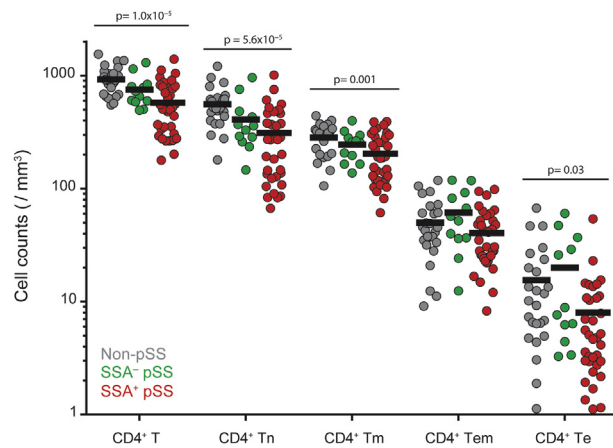


FIG E3. Absolute counts of most CD4⁺ T-cell subsets are decreased in patients with pSS. Absolute blood cell counts of the indicated cell subsets in each group of subjects are shown (see key). Significant *P* values are indicated (Mann-Whitney test). *Te*, Effector T; *Tem*, effector memory T; *Tm*, memory T; *Tn*, naive T.

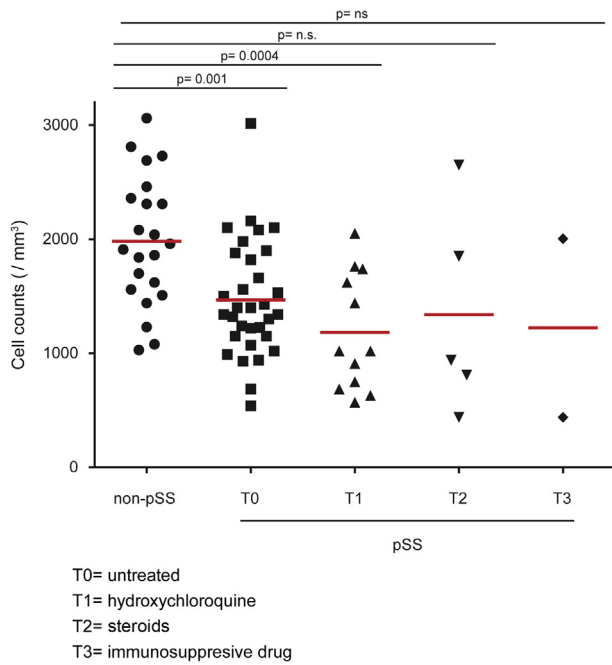


FIG E4. Lymphopenia is not caused by treatment in patients with pSS. Lymphocyte counts in each group of subjects are shown (see key). Significant *P* values are indicated (Mann-Whitney test). *ns*, Not significant.

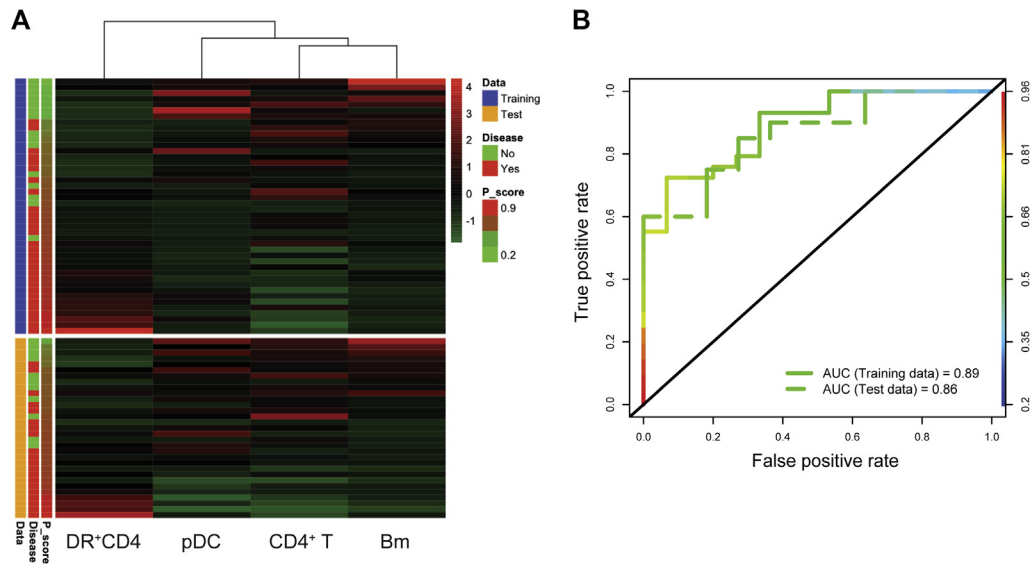


FIG E5. The blood pSS signature is a robust predictor of disease status. LASSO was used to select the most predictive subsets of cells and build a prediction model. **A**, Heat map of $\ln$ -transformed and standardized cell counts for the subsets of cells selected to predict pSS disease status. The prediction score (P_score) is color coded. Rows are ordered by the prediction score. **B**, Receiver operating characteristics curve of the prediction score from LASSO model for training and test data sets. *AUC*, Area under the curve.

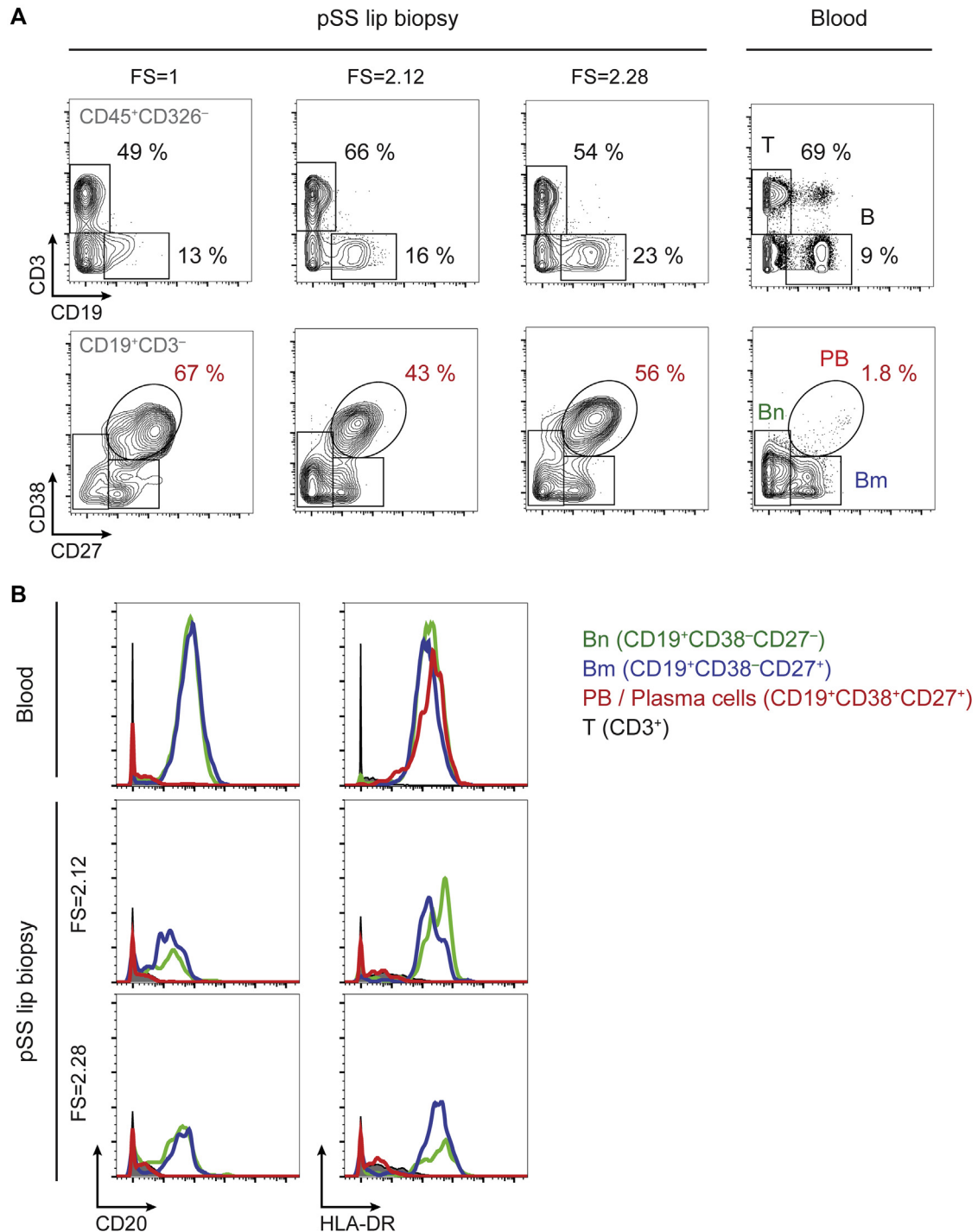


FIG E6. CD38⁺CD27⁺ cells detected in salivary gland biopsy specimens from patients with pSS are terminally differentiated plasma cells. **A**, Expression of CD3 and CD19 in CD45⁺ singlet cells (*top*) and CD38 and CD27 in CD19⁺ B cells (*bottom*) in peripheral blood and biopsy specimens from patients with pSS. Numbers indicate cell percentages in the outlined window. **B**, Expression of CD20 and HLA-DR in indicated cell subsets (see key). Naive and memory B cells detected in biopsy specimens were HLA-DR⁺, indicating that the absence of expression in plasma cells was not caused by the enzymatic digestion required to prepare single-cell suspensions. Bn, Naive B; PB, plasmablasts.

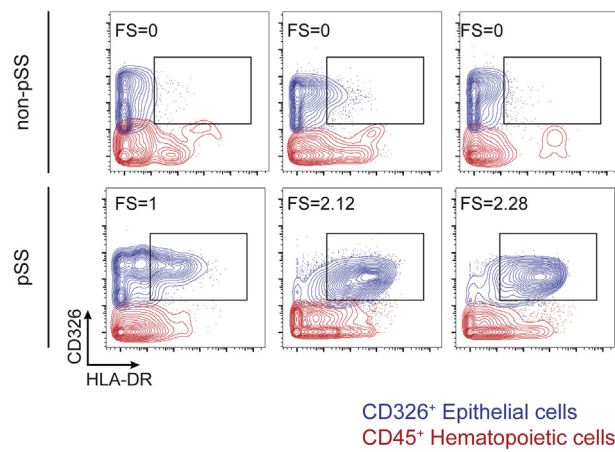


FIG E7. Selective upregulation of HLA-DR in glandular epithelium from patients with pSS. CyTOF analysis of CD326 and HLA-DR in epithelial (*blue*) and hematopoietic (*red*) cells from representative subjects without and with pSS.

TABLE E1. CyTOF immunophenotyping antibody panel

Antigen	Antibody clone	Symbol	Mass
CD45	HI30	Y	89
CD4	RPA-T4	Cd	114
CD326	9C4	Pr	141
CD19	HIB19	Nd	142
CD5	UCHT2	Nd	143
HLA-ABC	W6-32	Nd	144
CD80	L307	Nd	145
IgD	IA6-2	Nd	146
CD20	2H7	Sm	147
CD16	3G8	Nd	148
CD45RO	UCHL1	Sm	149
mDC8	DD-1	Nd	150
CD123	6H6	Eu	151
$\gamma\delta$ TCR	11F2	Sm	152
CD303	201A	Eu	153
CD1c	L161	Sm	154
CD86	IT2.2	Gd	156
CD27	L128	Gd	158
CCR7	G043H7	Tb	159
CD14	M5E2	Gd	160
CD11c	Bu15	Dy	162
CD161	HP-3G10	Dy	164
CD40	5C3	Ho	165
CD24	ML5	Er	166
CD38	HIT2	Er	167
CD8	SK1	Er	168
CD25	2A3	Tm	169
CD3	UCHT1	Er	170
CXCR5	RF8B2	Yb	171
IgM	MHM-88	Yb	172
CD141	1A4	Yb	173
HLA-DR	L243	Yb	174
CD279	EH12.2H7	Lu	175
CD56	NCAM16.2	Yb	176

TABLE E2. IHC/immunofluorescence antibody panel

Antigen	Species	Company	Clone	Concentration
CD45	Mouse monoclonal	Abcam, Cambridge, Mass	MEM-28	0.5 mg/mL
HLA-DR	Rabbit polyclonal	Abcam	NA (Cat#Ab137832)	0.98 mg/mL
CD8	Mouse monoclonal	Dako, Carpinteria, Calif	C8/144B	10 mg/mL
CD303	Mouse monoclonal	Dendritics, Lyon, France	124B3.13	0.8 mg/mL
CD138	Mouse monoclonal	Ventana, Tucson, Ariz	B-A38	0.13 mg/mL

TABLE E3. Demographic and clinical data for analysis of PBMCs

	Subjects without pSS	Patients with pSS
No. of subjects	45	49
Demographic data		
Women, no. (%)	35 (78)	48 (98)
Age (y), median (IQR)	53 (32.5-62)	54 (43.5-63.5)
Clinical data		
Anti-SSA ⁺ , no. (%)	0	35 (71)
Anti-SSB ⁺ , no. (%)	0	16 (33)
Lymphocyte count (cells/mm ³), median (IQR)	1910 (1560-2360)	1340 (1005-1835)
Gammaglobulinemia (g/L), median (IQR)	10.6 (9.2-12.2)	13.8 (10.4-17.9)
IgG level (g/L), median (IQR)	10.7 (9.3-12.3)	14.9 (10.9-18.3)
ESSDAI (median, [IQR], min, max)	NA	2 (1-4)
ESSDAI (0), no. (%)	NA	11 (22)
ESSDAI (1-4), no. (%)	NA	30 (61)
ESSDAI (≥5), no. (%)	NA	8 (16)
ESSDAI (≥14), no. (%)	NA	4 (8)
Treatment		
Steroids, no. (%)	0	5 (11.6)
Hydroxychloroquine, no. (%)	1 (2.2)	12 (27.9)
Methotrexate, no. (%)	0	0
Immunosuppressive drugs, no. (%)	0	2 (4.4)

TABLE E4. Demographic and clinical data for the analysis of biopsy specimens

	Subjects without pSS	Patients with pSS
No. of subjects	13	16
Demographic data		
Women, no. (%)	13 (100)	16 (100)
Age (y), median (IQR)	56 (41-60)	52.5 (34.25-59.5)
Clinical data		
Biopsy Chisholm grade	0 (0-1)	4 (2-4)
Biopsy FS	0	1.51 (0.5-2.09)
Anti-SSA ⁺ , no. (%)	0	13 (81.25)
Anti-SSB ⁺ , no. (%)	0	7 (43.75)
RF ⁺ , no. (%)	1 (7.7)	10 (6.25)
ESSDAI score, median (IQR)	NA	2.5 (0.25-4)
Treatment		
Steroids, no. (%)	0	3 (18.75)
Hydroxychloroquine, no. (%)	0	2 (12.5)
Methotrexate, no. (%)	0	0
Immunosuppressive drugs, no. (%)	0	0