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

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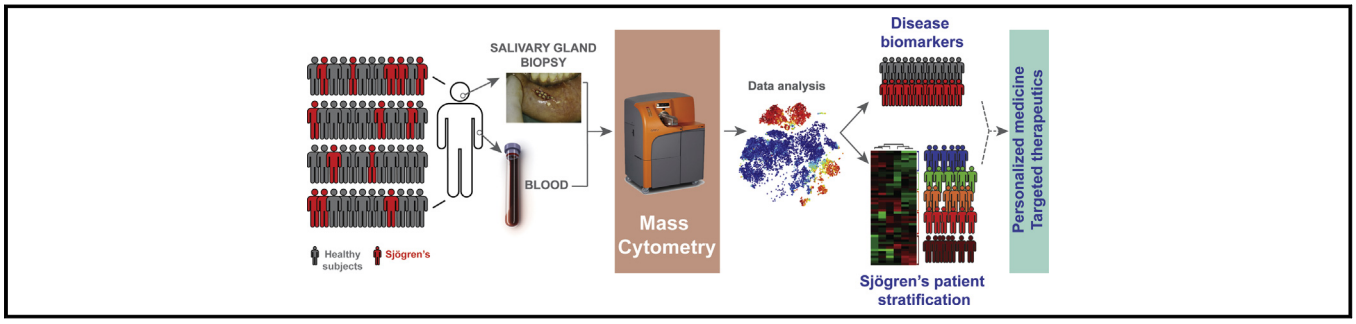
GRAPHICAL ABSTRACT



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*

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

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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 Mariette[3]). 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 al[6] 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.