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Molecular Subsetting of Interferon Pathways in Sjögren's Syndrome

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Objective. **Sjögren's syndrome (SS) is an autoimmune disease that targets the salivary and lacrimal glands. While all patients demonstrate inflammatory infiltration and abnormal secretory function in the target tissues, the disease features, pathology, and clinical course can vary. Activation of distinct inflammatory pathways may drive disease heterogeneity. The purpose of this study was to investigate whether activation of the interferon (IFN) pathway correlates with key phenotypic features.**

Methods. **Clinical data and 1 labial salivary gland (stored frozen) were obtained from each of 82 participants (53 patients with primary SS and 29 control subjects) in the Sjögren's International Collaborative Clinical Alliance (SICCA) registry. Salivary gland lysates were immunoblotted with markers of type I or type II IFN, and patterns of IFN activity were determined by hierarchical clustering. Correlations between SS phenotypic features and IFN activity in the salivary gland were performed.**

Results. **A total of 58% of the SS participants had high IFN activity and differed significantly from those with low IFN activity (higher prevalence of abnormal findings on sialometry, leukopenia, hyperglobulinemia, high-titer antinuclear antibody, anti-SSA, and high focus score on labial salivary gland [LSG] biopsy). Three distinct patterns of IFN were evident: type I–predominant, type II–predominant, and type I/II mixed IFN. These groups were clinically indistinguishable except for the LSG focus score, which was highest in those with type II–predominant IFN.**

Conclusion. **The SS phenotype includes distinct molecular subtypes, which are segregated by the magnitude and pattern of IFN responses. Associations between IFN pathways and disease activity suggest that IFNs are relevant therapeutic targets in SS. Patients with distinct patterns of high IFN activity are clinically similar, demonstrating that IFN-targeting therapies must be selected according to the specific pathway(s) that is active in vivo in the individual patient.**

Primary Sjögren's syndrome (SS) is a chronic autoimmune inflammatory disease that is characterized by lymphocytic infiltration of the salivary and lacrimal glands, resulting in abnormal tear and saliva secretion (1–3). Although all SS patients have abnormal secretory function and inflammatory infiltration of their salivary glands, there is significant heterogeneity in the disease features, pathology, and clinical course (4,5). This heterogeneity is a feature of all rheumatic autoimmune

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Drs. Hall, Rosen, and Casciola-Rosen have pending patent applications (P11883-03 US and P1183-04 Europe) for the use of specific markers of type II interferon activity to define the origin of interferon signatures in human rheumatic diseases. Dr. Baer has received consulting fees, speaking fees, and/or honoraria from Glenmark Pharmaceuticals (less than $10,000). Dr. Rosen has received consulting fees, speaking fees, and/or honoraria from MedImmune (less than $10,000).

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diseases and likely reflects distinct patient subsets within a primary disease phenotype, as driven by unique pathophysiologic mechanisms.

While substantial evidence indicates that interferons (IFNs) play significant roles in the pathogenesis of rheumatic diseases, including SS (6–13), there is striking heterogeneity in IFN activity among individuals and among diseases. Indeed, it remains to be determined whether type I or type II IFNs are the primary drivers of the IFN signature seen in patients with SS and other rheumatic diseases (14) and whether IFN expression in target tissues is associated with disease activity. In recent studies (12), we defined and validated specific markers of type I and type II IFN activity and used these probes in a small study to investigate the distinct IFN pathways that are active in patient tissues. We examined relevant target tissues in patients with SS and dermatomyositis and determined that different patterns of IFN activity were apparent in different rheumatic diseases and that the magnitude of the IFN effects varied significantly among the patients.

While there is heterogeneity of the IFN signatures in SS, the frequency and clinical associations of the different patterns are unclear. To better understand this, we investigated the IFN expression patterns in labial salivary glands (LSGs) from a large cohort of well-characterized SS patients and controls. All subjects were enrolled in the Sjögren's International Collaborative Clinical Alliance (SICCA) registry, which systematically collected extensive phenotype data and biologic specimens across 9 international research sites between 2003 and 2013 (15). Based on our recent findings (12), we selected IFN-induced protein with tetratricopeptide repeats 3 (IFIT-3) to use for readout of type I IFN, with IFN-inducible guanylate binding protein 1 (GBP-1) and GBP-2 to use as markers of type II IFN activity (for immunoblotting and immunohistochemistry, respectively) in the current study. We show that high levels of IFN activity are associated with a more severe disease phenotype and that distinct IFN patterns are apparent in the group with high IFN activity. Although SS patients in this group are clinically indistinguishable, those with type II IFN activity have higher LSG focus scores, and the presence of inflammatory infiltrates correlates well with type II, but not type I, IFN activity.

As therapies targeting immune effector pathways become increasingly available, it will be helpful to develop approaches that quantitatively define inflammatory pathway activity in patient tissues in order to assess their activity prior to initiating treatment. These studies demonstrate that analysis of patient-derived target tissues can identify distinct molecular subgroups. These analyses provide opportunities to identify optimal candidates for participation in clinical trials, to monitor therapeutic responses, and to determine the efficacy of novel agents in SS and possibly other autoimmune rheumatic diseases.

PATIENTS AND METHODS

Study participants. A single frozen LSG and corresponding clinical data were obtained from each of 82 participants in the SICCA registry (16). Paraffin-embedded salivary gland sections were obtained from a subset of 6 of these SICCA participants. SICCA participants underwent an LSG biopsy that was independently examined by 2 histopathologists (17) and had the phenotypic characteristics shown in Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39204/abstract); 53 participants had SS, as defined by American College of Rheumatology (ACR) criteria (18).

For the SS patient group in the present study, we selected SICCA participants who had a diagnosis of focal lymphocytic sialadenitis in an LSG biopsy sample, with a focus score of $\geq$1 (i.e., $\geq$1 focus/4 mm^2). We also selected the SS patients to represent a broad range of salivary gland lymphocytic infiltration, from mild to severe, which was defined arbitrarily by focus scores of 1 to $<$2 (mild), 2 to $<$3 (moderate), and $\geq$3 (severe). A group of 14 SICCA participants who had dry eye disease (ocular surface staining [OSS] score of $\geq$3 in either eye) occurring in the absence of serologic (either anti-SSA/SSB antibodies or an antinuclear antibody [ANA] titer of $\geq$1:320 plus rheumatoid factor) and histopathologic (no focal lymphocytic sialadenitis) evidence of SS served as a "non-SS dry-eye disease" control group. A group of 15 SICCA participants who had symptoms suggestive of SS but had no objective evidence of dry eyes (OSS score of $<$3 in both eyes), no focal lymphocytic sialadenitis, and negative findings on serologic studies (neither anti-SSA/SSB antibodies nor an ANA titer of $\geq$1:320 plus rheumatoid factor) served as a "non-SS no dry-eye disease" control group.

Participants with confirmed diagnoses of rheumatoid arthritis, systemic lupus erythematosus (SLE), and other autoimmune connective tissue diseases were excluded from the present study. None of the study subjects had evidence of lymphoma or hepatitis C.

Informed consent was obtained from all study participants, in compliance with the Declaration of Helsinki. The SICCA registry was approved by the institutional review boards of the study center (University of California, San Francisco) and each participating research site.

Laboratory testing. Complete blood cell counts were performed at the local research site, and all other laboratory testing of SICCA registrants was performed centrally by Quest Diagnostics. This included testing for ANA, anti-SSA antibody, anti-SSB antibody, rheumatoid factor, quantitative immunoglobulin levels, and C4 levels.

Immunoblotting. Frozen labial salivary glands were thawed on ice and homogenized in buffer containing Nonidet P-40, 20 m*M* Tris, pH 7.4, 150 m*M* NaCl, 1 m*M* EDTA, and a protease inhibitor cocktail, as described previously (19). Equivalent amounts of protein (4 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and immunoblotted with antibodies against GBP-1 (Santa Cruz Biotechnology) and IFIT-3 (Sigma-Aldrich), which we previously validated as precise probes of distinct IFN pathways (12). CD45

(BD Transduction Laboratories) was used as a pan-leukocyte marker, and vinculin (Sigma-Aldrich) and β-actin (Sigma-Aldrich) were used as loading controls. Horseradish peroxidase (HRP)–conjugated secondary antibodies (Jackson ImmunoResearch) were used for visualization and were developed with an enhanced chemiluminescence detection system (Pierce). A calibrator sample (lysates made from IFN-treated cultured human salivary gland cells for IFIT-3 and GBP-1, or for CD45, peripheral blood mononuclear cells from a healthy donor) was included on each gel for normalizing exposure times to ensure accurate quantitation across gels.

For densitometry, x-ray films were scanned with an AGFA Arcus II scanner, and densities were quantified using Bio-Rad Quantity One software. To define patterns of IFN-induced protein expression in an unbiased manner in individual patients, loading control–normalized expression values were median-centered, subjected to unsupervised hierarchical clustering in GenePattern (Broad Institute) using the Hierarchical Clustering algorithm (20), and visualized using JavaTreeView software (21).

Immunohistochemistry. Paraffin-embedded sections of LSGs from SS patients were processed for immunohistochemistry as described elsewhere (12). Briefly, after rehydration, antigen retrieval, and blocking, sections were incubated overnight at 4°C with antibodies against IFIT-3 (12.5 μg/ml; Novus Biologicals) or GBP-2 (30 μg/ml; Novus Biologicals). Incubations with HRP-conjugated secondary antibody were performed for 1 hour at room temperature, and staining was visualized with diaminobenzidine (Dako) according to the manufacturer's directions. Nuclei were counterstained with Mayer's hematoxylin. All images were captured using a Zeiss Axioskop 50 microscope equipped with a Zeiss AxioCam HRc camera and AxioVision 4 software.

Statistical analysis. Descriptive statistics were used to describe the demographic features of the study participants. We used a cross-sectional study design to investigate the correlation of IFN protein expression with SS clinical phenotype. Differences in phenotype characteristics were compared between patients with SS and controls, between the 2 control groups (non-SS dry eye disease and non-SS no dry eye disease), between SS patients with high versus low IFN activity in salivary gland lysates, and between SS patients with predominantly type I, type II, or a mixed type I/II IFN signature. Differences in categorical variables were assessed using Fisher's exact test, and differences in continuous variables by Wilcoxon's rank sum test or Kruskal-Wallis test as appropriate. Because of the exploratory nature of these analyses, no formal adjustment was made for multiple hypothesis testing.

We hypothesized that IFN activity was primarily related to the degree of salivary gland lymphocytic infiltration. To test this, we performed simple and multivariable logistic regression analyses to explore the explanatory role of key phenotypic features of SS in relation to the outcome of high versus low IFN activity in the salivary gland. Covariates examined included the following histologic, serologic, and clinical measures of disease activity based on existing literature: focus score, hyperglobulinemia, positive serologic findings as defined by the ACR classification criteria (anti-SSA or anti-SSB antibodies or ANA at a titer of ≥1:320 plus rheumatoid factor), ocular surface staining score, and salivary flow rate (15,17).

All statistical analyses were performed using JMP and Stata version 13 software.

RESULTS

Characteristics of the SS patients and control subjects. We obtained a single frozen LSG and corresponding clinical data from each of 82 participants in the SICCA registry (16). These consisted of 53 patients with SS and 29 control subjects (either non-SS dry eye disease [n = 14] or non-SS no dry eye disease [n = 15]). The de-

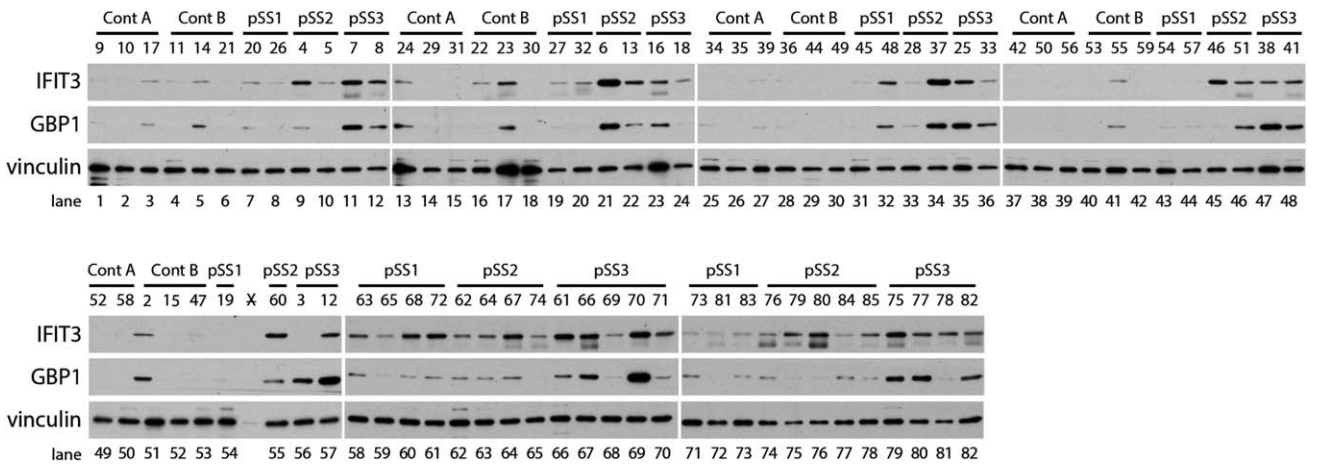

Figure 1. Distinct patterns of interferon (IFN) activity in lysates of labial salivary gland (LSG) biopsy samples from patients with primary Sjögren's syndrome (SS). Protein lysates prepared from LSG biopsy samples from control subjects (n = 29) or SS patients (n = 53) were probed for IFN activity by Western blotting. A marker of type II IFN (IFN-inducible guanylate binding protein 1 [GBP-1]) and a marker of type I IFN (IFN-induced protein with tetratricopeptide repeats 3 [IFIT-3]) were included. Vinculin was analyzed as a loading control. Patients with primary SS were categorized according to the degree of salivary gland lymphocyte infiltration: mild (focus scores of 1 to <2 [pSS1]), moderate (focus scores of 2 to <3 [pSS2]), or severe (focus score of ≥3 [pSS3]). Two groups of control subjects were evaluated: 14 with non-SS dry-eye disease (Cont A) and 15 with non-SS no dry-eye disease (Cont B) (see Patients and Methods for details). X indicates not enough protein to analyze or no protein loaded.

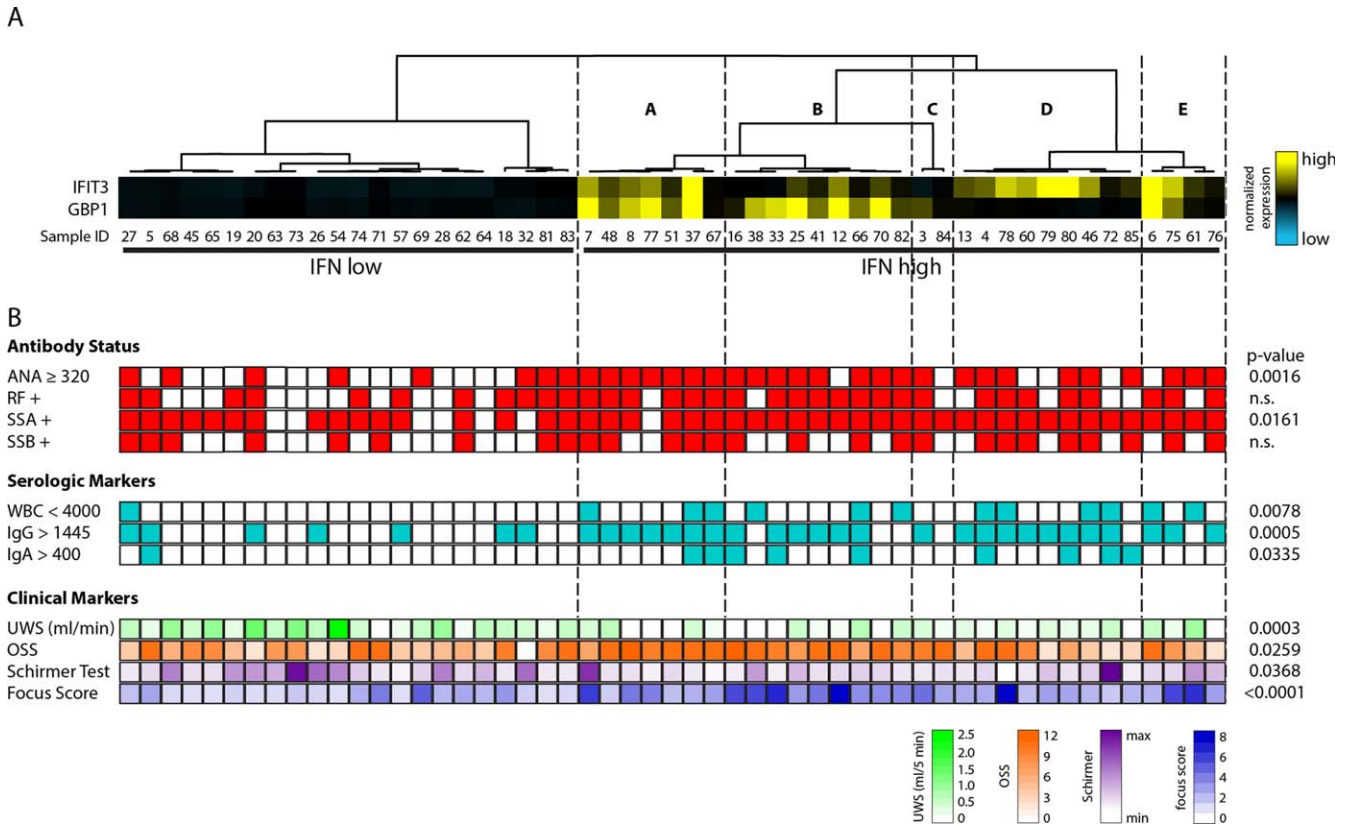

Figure 2. Correlation of clinical features with IFN activity in LSG biopsy samples from SS patients. **A,** IFN-induced protein expression in SS patients shown in Figure 1, as quantified by densitometry and normalized to the level of vinculin expression in the same sample. Vinculin-normalized expression values were subjected to unsupervised hierarchical clustering to define patterns of IFN activity in each patient. Patients with high IFN activity had different activity patterns, as delineated by the vertical broken lines labeled A–E (see Results for details). **B,** The clinical features (antibody status, serologic markers, and clinical markers) of each SS patient. The clinical features are aligned with the respective clustering data. White blocks represent negative values, and colored blocks represent positive values. Units of measure for the serologic markers are as follows: white blood cells (WBC) <4,000/μl, IgG >1,445 mg/dl, and IgA >400 mg/dl. *P* values represent low IFN activity versus high IFN activity. ANA = antinuclear antibody; RF = rheumatoid factor; UWS = unstimulated whole salivary flow rate; OSS = ocular surface staining score; NS = not significant (see Figure 1 for other definitions).

mographic and key phenotypic features of the 82 study subjects are shown in Supplementary Table 1; the features of the 2 control populations are shown separately in Supplementary Table 2 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39204/abstract). The SS and control subject groups did not differ significantly in terms of age, sex, or ethnicity. Of the 29 controls, 14 had idiopathic dry eye disease (see Patients and Methods for details). Since the OSS score is the single feature that distinguishes the 2 control groups, we combined these 29 individuals into a single group for all subsequent studies.

The majority of the SS patients were women, with a median age of 56 years. The ethnic distribution of the cohort reflected the global nature of the SICCA registry. As expected, the majority of SS patients had symptoms of dry eyes and dry mouth, an OSS score ≥3, unstimulated whole salivary flow (UWSF) rate <0.5 ml/5 minutes, hyperglobulinemia, as well as high-titer ANA, rheumatoid factor, and SSA and/or SSB antibodies.

Heterogeneity of IFN activity in LSG biopsy samples from the SS patients. In a recent study, we extensively validated probes specific for type I or type II IFN pathways and addressed the complexity of the system in great detail (12). We used these well-defined probes as tools in the present study to quantitatively analyze salivary gland biopsy lysates obtained from 53 SS patients and 29 controls (a single frozen gland was used to generate each lysate). Equivalent amounts of SS patient and control salivary gland protein lysates were separated by SDS-PAGE and immunoblotted with antibodies against IFIT-3 or GBP-1, probes which read out type I and type II IFN activity, respectively. Data from all patients and controls are presented in Figure 1.

To address the relationship between glandular lymphocytic infiltration and IFN activity, SS patients were selected for analysis based on focus scores (see Patients and Methods). Interestingly, higher levels of IFN-induced protein expression were evident in patients with higher salivary gland focus scores (moderate and severe groups). While most exhibited evidence of type I and type II IFN activity concomitantly (Figure 1), patterns consistent with type I IFN–predominant (samples 46, 60, 79, and 80) and type II IFN–predominant (samples 33 and 3) responses were also apparent. Compared to tissues from SS patients, the levels of IFN-inducible protein expression were low or absent in all salivary gland lysates from control subjects.

In order to objectively classify IFN expression in SS biopsy samples, the data were quantified by densitometry, and the expression of IFIT-3 and GBP-1 was normalized to the level of vinculin (the loading control) in the same sample. The data were median-centered and subjected to unsupervised hierarchical clustering. Two major subgroups were identified based on IFN pathway activity: those with low IFN activity and those with high IFN activity (Figure 2A). This unbiased approach showed robust IFN pathway activity in 31 of 53 SS patients (58%). Evidence of IFN activity was robust in most samples, while others (e.g., samples 76 and 84) clustered with the IFN high group but had levels that were only modestly above those in the IFN low group (see Figure 1). Among the patients with high IFN activity, different activity patterns were evident, with 9 of 31 (29%) demonstrating a type I IFN–predominant pattern (Figure 2A, branch D), 11 of 31 (35.5%) having a type II IFN-predominant pattern (Figure 2A, branches B and C), and 11 of 31 (35.5%) having evidence of type I and type II IFN activity (Figure 2A, branches A and E).

Immunohistochemical evidence of IFN pathway heterogeneity in patient tissues. We next determined whether the 3 patterns defined by immunoblotting (type I IFN–predominant, type II IFN–predominant, and type I and type II mixed IFN) were evident by immunohistochemistry in tissue biopsy samples. For this, we selected representative patients from each of the 3 distinct biochemically defined IFN patterns. Since we previously showed that markers of these IFN-induced proteins did not stain LSG from control subjects, we focused our immunohistochemical comparison on the SS patient spectrum described above.

Serial paraffin sections of LSG biopsy samples were stained with antibodies against IFIT-3 and GBP-2 (both previously validated and used as markers of types I and II IFN activity, respectively, on paraffin-embedded LSG sections [12]). The immunohistochemical staining

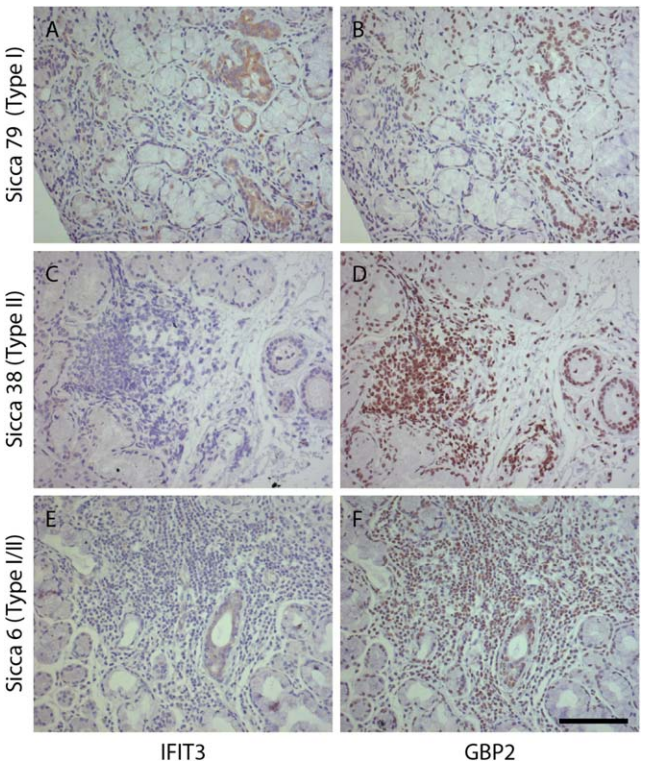

Figure 3. Distinct patterns of IFN activity in paraffin-embedded sections of LSGs from SS patients. LSG biopsy samples from 6 SS patients, each with distinct biochemically defined patterns of IFN activity (type I–predominant, type II–predominant, and types I and II mixed), were stained with antibodies against IFIT-3 (**A, C,** and **E**) or GBP-2 (**B, D,** and **F**). Representative images from a patient with each pattern (sicca patients 79, 38, and 6) are shown. Bar = 100 μm. See Figure 1 for definitions.

patterns correlated with our biochemical findings and were consistent with our previously reported observations (12). Representative data from patients with type I IFN–predominant (patient 79; lane 75 in Figure 1), type II IFN–predominant (patient 38; lane 47 in Figure 1), and types I and II mixed IFN activity (patient 6; lane 21 in Figure 1) are shown in Figure 3.

IFIT-3 staining (Figures 3A, C, and E) was seen in salivary duct epithelial cells, while GBP-2 staining (Figures 3B, D, and F) was prominent within the nuclei of lymphoid cells and duct epithelia, which were surrounded by inflammatory infiltrates. The most pronounced IFIT-3 staining was seen in a patient identified as type I IFN–predominant in regions without significant infiltrates (Figure 3A), suggestive of a plasmacytoid dendritic cell–driven IFN process. Consistent with the biochemical findings, IFIT-3 staining was not detected in salivary gland biopsy samples from patients with the type II IFN pattern (Figure 3C). In contrast, GBP-2 was robust in type II and type I/II IFN tissues, where it was

Table 1. Comparison of demographic and phenotypic characteristics of 53 SS patients, according to the level of IFN activity*

SS characteristic	High IFN activity (n = 31)	Low IFN activity (n = 22)	*P*
Categorical variables†			
Female	28 (90)	19 (86)	0.683
Caucasian	17 (55)	14 (64)	0.581
Asian/Pacific Islander	9 (29)	5 (23)	0.755
WBCs <4,000/μl	12 (39)	1 (5)	0.008
IgG >1,445 mg/dl	25 (81)	7 (32)	0.0005
IgA >400 mg/dl	9 (29)	1 (5)	0.034
ANA titer ≥1:320	25 (81)	8 (36)	0.002
Rheumatoid factor	23 (74)	11 (50)	0.088
Anti-SSA	30 (97)	16 (73)	0.016
Anti-SSB	18 (58)	9 (41)	0.271
C4 <16 mg/dl	7 (23)	7 (32)	0.534
Dry eye symptoms	30 (97)	17 (77)	0.071
Dry mouth symptoms	30 (97)	20 (91)	0.563
Continuous variables‡			
Age, years	56 (45–64)	53.5 (43.5–62.25)	0.56
LSG focus score	3.1 (2.4–5.7)	1.45 (1.08–2.55)	<0.0001
UWSF rate, ml/5 minutes	0.164 (0–0.415)	0.549 (0.256–0.978)	0.0003
OSS score, maximum of both eyes	10 (7–11)	6 (4–9.25)	0.026
Schirmer's test, mm/5 minutes, mean of both eyes	4 (3–6.5)	6.5 (3.5–16.6)	0.037

* SS = Sjögren's syndrome; IFN = interferon; WBCs = white blood cells; ANA = antinuclear antibody; LSG = labial salivary gland (focus score ≥1, representing ≥1 focus/4 mm^2); UWSF = unstimulated whole salivary flow (dry mouth defined as rate of <0.5 ml/5 minutes); OSS = ocular surface staining (dry eyes defined as score of ≥3 in either eye).
† Categorical variables are shown as the number (%). Statistical analyses were performed with Fisher's exact test.
‡ Continuous variables are shown as the median (interquartile range). Statistical analyses were performed with Wilcoxon's rank sum test.

prominent in the nuclei of infiltrating inflammatory cells and duct epithelium, but was seen only at low levels in type I IFN tissue.

Clinical characterization of SS patients with activated IFN pathways. To determine whether specific phenotypic characteristics of SS were associated with IFN activity, we compared clinical measures of disease activity between SS patients with high versus low IFN activity (Table 1). The high IFN group had lower UWSF rates (median 0.164 ml/5 minutes versus 0.549 ml/5 minutes; $P = 0.0003$), higher maximum OSS scores (median 10 versus 6; $P = 0.0259$), and lower mean values on Schirmer's test (median for both eyes 4 mm/5 minutes versus 6.5 mm/5 minutes; $P = 0.0368$), indicating an overall greater disruption of secretory function in IFN-positive participants. In addition, laboratory and serologic markers, which were more prevalent among the high IFN group, included high-titer ANA (≥1:320; 81% versus 36% [$P = 0.0016$]), and anti-SSA antibodies (97% versus 73%; $P = 0.0161$), hyperglobulinemia (IgG >1,445 mg/dl; 81% versus 32% [$P = 0.0005$] and IgA >400 mg/dl; 29% versus 5% [$P = 0.0335$]), and leukopenia (WBCs <4,000/μl; 39% versus 5% [$P = 0.0078$]). The focus score was also significantly higher in the group with high IFN activity (median 3.1 versus 1.45; $P < 0.0001$). Significant findings in individual patients are presented in Figure 2B.

We postulated that high IFN activity was determined primarily by glandular lymphocytic infiltration, as measured semiquantitatively by the focus score. To test this hypothesis and to examine whether other factors remained predictive of high versus low IFN activity after adjusting for focus scores, we fitted a logistic regression model using variables that we considered to be most relevant as markers of disease activity, based on the literature. These included the focus score, hyperglobulinemia, positive serology as defined by the ACR classification criteria (anti-SSA or anti-SSB antibodies or ANA titer ≥1:320 plus rheumatoid factor), the OSS score, and the UWSF rate. In the adjusted model, both the focus score (odds ratio 3.3 [95% confidence interval 1.3–8.4]) and IgG hyperglobulinemia (odds ratio 12.1 [95% confidence interval 1.7–85.5]) were statistically significant predictors of high glandular IFN activity (see Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39204/abstract).

To determine whether patterns of IFN activity correlate with clinical features of the disease, we stratified the 31 IFN-positive SS patients into the 3 predominant IFN

Table 2. Comparison of demographic and phenotypic features of 31 SS patients, according to predominant type I, type II, or type I/II mixed IFN activity*

SS feature	Type I IFN (n = 9)	Type II IFN (n = 11)	Type I and type II mixed IFN (n = 11)
Categorical variables†			
Female	9 (100)	10 (91)	9 (82)
Caucasian	6 (67)	4 (36)	7 (64)
Asian/Pacific Islander	3 (33)	4 (36)	2 (18)
WBCs <4,000/μl	4 (44)	3 (27)	5 (45)
IgG >1,445 mg/dl	8 (89)	7 (64)	10 (91)
IgA >400 mg/dl	4 (44)	3 (27)	2 (18)
ANA titer ≥1:320	6 (67)	9 (82)	10 (91)
Rheumatoid factor	5 (56)	9 (82)	9 (82)
Anti-SSA	9 (100)	11 (100)	10 (91)
Anti-SSB	6 (67)	5 (45)	7 (64)
C4 <16 mg/dl	0	5 (45)	2 (18)
Dry eye symptoms	9 (100)	10 (91)	11 (100)
Dry mouth symptoms	9 (100)	10 (91)	11 (100)
Continuous variables‡			
Age, years	52 (45–69.5)	58 (52–64)	56 (39–63)
LSG focus score	2.6 (2.1–2.85)	4.3 (3.5–4.7)§	2.9 (2.3–5.8)
UWSF rate, ml/5 minutes	0.229 (0.0975–0.297)	0.114 (0–0.491)	0.164 (0–0.552)
OSS score, maximum of both eyes	5 (2.5–10)	11 (9–11)¶	11 (7–11)
Schirmer's test, ml/5 minutes, mean of both eyes	3.75 (2.625–6.25)	4 (3–6.5)	5 (3–9)

* SS = Sjögren's syndrome; WBCs = white blood cells; ANA = antinuclear antibody; LSG = labial salivary gland (focus score ≥1, representing ≥1 focus/4 mm^2); UWSF = unstimulated whole salivary flow (dry mouth defined as rate of <0.5 ml/5 minutes); OSS = ocular surface staining (dry eyes defined as score of ≥3 in either eye).
† Values for categorical variables are the number (%). Statistical analyses performed with Fisher's exact test.
‡ Values for continuous variables are the median (interquartile range). Statistical analyses performed with the Kruskal-Wallis test.
§ $P = 0.024$ versus the type I interferon (IFN) group.
¶ $P = 0.042$ versus the type I IFN group.

pathway groups: type I IFN predominant (n = 9), type II IFN predominant (n = 11), and types I and II mixed IFN (n = 11). We then compared the expression of key phenotypic features between the groups (Table 2). Although the total numbers of patients in the 3 groups were small, it is noteworthy that the differences in focus scores reached statistical significance, with the lowest focus scores in the type I IFN–predominant group and highest in the type II IFN–predominant group ($P = 0.024$ by Kruskal-Wallis test). Two additional measures of disease activity were different across the 3 groups: the frequency of C4 hypocomplementemia ($P = 0.0488$) and the OSS score ($P = 0.0416$), albeit with marginal levels of statistical significance. C4 hypocomplementemia was more frequent in the type II IFN group.

Association of type II IFN activity with the presence of CD45+ infiltrates. To directly analyze associations between inflammation and IFN activity, we interrogated the aggregate level of inflammation in each tissue. Since the components of the inflammatory infiltrate in SS tissues are heterogeneous (22), we analyzed the expression of a pan-leukocyte marker, CD45, in each sample by immunoblotting (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39204/abstract). There was striking heterogeneity in CD45 protein expression among the SS patients. CD45 expression was normalized to the level of β-actin (included as a loading control) in the same sample, and the population was divided into tertiles of CD45 expression for analysis. We compared normalized CD45 expression with normalized IFIT-3 or GBP-1 in each patient. GBP-1 levels were highest in patients with the greatest CD45 expression (Figure 4A). However, IFIT-3 expression could be found at high levels in both the absence and presence of CD45 expression (Figure 4B).

DISCUSSION

The significant heterogeneity among SS patients complicates disease classification, assignment of disease mechanism, and selection of therapy. These challenges underscore the need to discover novel approaches to the classification of disease pathophysiology. As treatment for SS and other rheumatic diseases moves toward a personalized approach, developing tools that can reliably define inflammatory pathway activity is a major priority. In this study, we quantified the patterns of

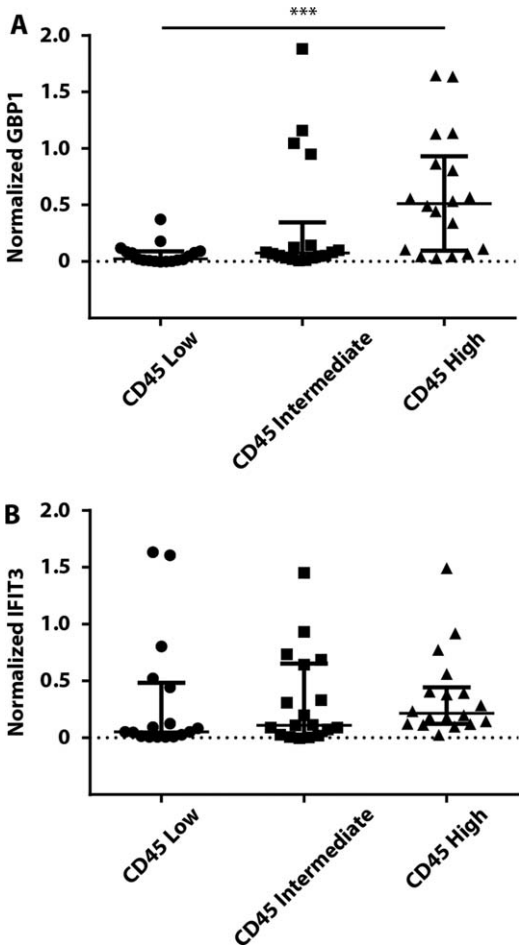

Figure 4. Association between CD45+ infiltrates in LSG tissues with high type II–predominant IFN activity from 53 SS patients. CD45 expression (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39204/abstract) was quantified by densitometry and normalized to the level of β-actin in the same sample. Patients were divided into tertiles based on normalized levels of CD45 expression: low (n = 17), intermediate (n = 18), and high (n = 18). Normalized expression (shown as normalized OD units) of GBP-1 (**A**) and IFIT-3 (**B**) were compared for each CD45 group. Dotted line is an extension of the 0 value. Each symbol represents an individual patient; bars show the median and interquartile range. *** = $P = 0.0004$ by Kruskal-Wallis test. See Figure 1 for definitions.

IFN activity in LSGs from a large cohort of well-characterized SS patients and controls without SS to determine whether IFN activity was associated with clinical phenotypes. We were able to achieve this using a single frozen LSG from each participant.

We demonstrated that IFN activity was high in 31 of 53 SS patients and was associated with a more severe disease phenotype (characterized by more prevalent salivary hypofunction and ocular dryness, a higher focus score, leukopenia, SSA antibodies, high-titer ANA, and hyperglobulinemia). Both the focus score and hyperglobulinemia were the most significant predictors of high IFN activity in a multivariate model adjusted for the effects of the focus score, positive findings on SS serology, the OSS score, the UWSF rate, and hyperglobulinemia.

The patterns of IFN activity were heterogeneous, and SS patients in the high IFN group could be further stratified by the IFN pathways that were most active in their salivary gland tissue, including type I–predominant, type II–predominant, and mixed type I/II IFN activity. Interestingly, these patients were indistinguishable in their key SS phenotypic features except for the focus score, which was highest in type II–predominant patients. The lack of a difference in clinical phenotype between these different IFN patterns could be due to our relatively small sample size and the imprecision of certain clinical measures; however, the lack of differentiating clinical features between different IFN patterns suggests the need to interrogate inflammatory pathways directly in target tissues to determine which pathways are active. Our data showed that subsets based on molecular signatures enriched in LSGs can be defined, thus providing a quantitative, standardizable approach to the classification of inflammatory pathway activity in patients' tissues. The definition of patient subsets within a group of patients having a similar clinical profile may potentially be useful in the setting of disease treatment.

The focus score is a count of discrete lymphocytic infiltrates normalized to 4 mm^2 of gland tissue. It does not measure the percentage of gland tissue infiltrated by lymphocytes and is therefore not an accurate measure of total glandular inflammation. In contrast, analysis of whole glands/tissues (rather than tissue sections, as is the case with immunohistochemical scoring of disease in tissue) enables in vivo events to be viewed in aggregate. Whole-gland biochemistry also provides a more integrated analysis of gland tissue, with areas of the gland that were not examined histologically included in the biochemical analysis. Expression of proteins will also be influenced by salivary gland tissue heterogeneity among SS patients, including the extent and nature of the infiltrate, the amount of epithelial structure destruction, healing, and replacement of glandular tissue by fat or fibrosis. Biochemical analysis of whole glands integrates these additional sources of variation, and future studies using specific markers will also allow these additional processes to be analyzed.

To date, most clinical trial selection criteria in the rheumatic diseases have been based on broad phenotypic features, and the results of these trials have not

been striking in terms of clinical response (23–25). Defining the activity of inflammatory pathways in disease-relevant target tissues prior to initiating a controlled clinical trial and examining the response of such pathways to therapy may provide important stratification tools and pharmacodynamic markers. Similar approaches have provided important tools for the study of novel cancer therapies, where inclusion in a clinical trial requires the presence of a genetically defined marker (e.g., *ALK* gene rearrangements, *BRAF* V600E mutation, *HER2/neu*) which identifies the active pathway (26–28).

Our data demonstrate that significant heterogeneity occurs in IFN pathway activation in SS patients. The approach defined here for the quantification of inflammatory pathways in tissues uses tiny amounts of patient material; 4 μg of protein lysate was sufficient to assay the relevant proteins. Using such tools to stratify patients and to select therapies could provide a novel method for selecting patients for clinical trials and could thus improve the chances of identifying disease subgroups in which specific IFN inhibition might be beneficial. Of note, this approach is readily applicable to other inflammatory pathways and autoimmune diseases, especially those with well-defined, accessible target tissues.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Casciola-Rosen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Hall, Baer, Shiboski, Rosen, Casciola-Rosen.

Acquisition of data. Hall, Baer, Criswell, Shiboski, Casciola-Rosen.

Analysis and interpretation of data. Hall, Baer, Shah, Rosen, Casciola-Rosen.

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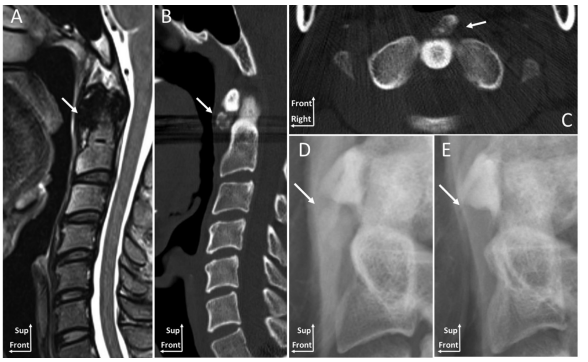
Clinical Images: Acute calcific tendinitis of the longus colli muscle



The patient, a 32-year-old man, presented with a 1-month history of severe pain in the posterior neck and shoulder without evidence of any trigger. He also experienced mild odynophagia while drinking water. Physical examination revealed a markedly reduced active range of cervical motion. No fever was noted, and laboratory tests revealed a normal complete blood cell count, erythrocyte sedimentation rate, and C-reactive protein level. Sagittal T2-weighted magnetic resonance imaging of the cervical spine revealed a low-intensity nodular lesion around the C2 body (**arrow** in **A**); computed tomography (CT) of the neck revealed a large, oval calcification, inferior to the anterior arch of the C1 body (**arrows** in **B** and **C**). Thus, the patient was diagnosed as having calcific tendinitis of the longus colli muscle. The symptoms quickly resolved following therapy with oral nonsteroidal antiinflammatory drugs and colchicine. Lateral cervical radiographs taken after 4 months confirmed the complete resolution of the calcification (**arrows** in **D** and **E**). Acute calcific tendinitis of the longus colli muscle (or retropharyngeal tendinitis) is an aseptic inflammatory process caused by calcium hydroxyapatite crystal deposition in the longus colli tendon. It is typically observed between ages 30 and 60 years (1), and presents as neck pain, limited neck movement, and dysphagia or odynophagia (2). During diagnosis, this condition should be differentiated from retropharyngeal abscess, cervical osteomyelitis, spondylodiscitis, retro-odontoid pseudotumor, crowned dens syndrome, and meningitis. CT is the most sensitive diagnostic imaging technique and enables the identification of the amorphous calcification in the proximal fibers of the longus colli—a specific characteristic of this disease (3).

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