Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome

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Gene expression analysis of target organs might help provide new insights into the pathogenesis of autoimmune diseases. We used global gene expression profiling of minor salivary glands to identify patterns of gene expression in patients with primary Sjögren's syndrome (pSS), a common and prototypic systemic autoimmune disease. Gene expression analysis allowed for differentiating most patients with pSS from controls. The expression of 23 genes in the IFN pathways, including two Toll-like receptors (TLR8 and TLR9), was significantly different between patients and controls. Furthermore, the increased expression of IFN-inducible genes, BAFF and IFN-induced transmembrane protein 1, was also demonstrated in ocular epithelial cells by quantitative RT-PCR. *In vitro* activation showed that these genes were effectively modulated by IFNs in salivary gland epithelial cells, the target cells of autoimmunity in pSS. The activation of IFN pathways led us to investigate whether plasmacytoid dendritic cells were recruited in salivary glands. These IFN-producing cells were detected by immunohistochemistry in all patients with pSS, whereas none was observed in controls. In conclusion, our results support the pathogenic interaction between the innate and adaptive immune system in pSS. The persistence of the IFN signature might be related to a vicious circle, in which the environment interacts with genetic factors to drive the stimulation of salivary TLRs.

autoimmune disease | innate immunity | salivary gland

Sjögren's syndrome (SS) is a common systemic autoimmune disease that can be primary or occur in association with other autoimmune diseases. The prevalence of SS is second only to that of rheumatoid arthritis. This disease preferentially involves salivary and lacrimal glands, leading to the main symptoms of ocular and mouth dryness, but a wide range of other organs might be targeted (1). Moreover, primary SS (pSS) can be used as a prototypic disease for investigating the relation between autoimmunity and lymphoma, because 5% of patients with pSS develop B cell non-Hodgkin's lymphoma (2, 3).

pSS is currently considered a complex disease involving environmental and genetic factors (4–6). However, the mechanisms driving the autoimmune process in pSS have not been unraveled, despite extensive studies. The absence of familial clustering of the disease (in contrast with that of other autoimmune diseases) precludes gene-mapping efforts. Until recently, no experimental model was particularly relevant concerning the pathogenesis of pSS. Thus, genome-wide gene expression profiling involving microarrays could be a good strategy to study the pathogenesis of this disease. This approach, which allows the simultaneous analysis of thousands of mRNA transcripts in a biologic sample, has been applied successfully in the investigation of various human malignant diseases (7). In the field of autoimmune diseases, most of the microarray studies

used gene expression profiling of peripheral blood mononuclear cells (8, 9). Given the predominant organ specificity in pSS, we explored the gene expression of minor salivary gland tissue, a target organ of the disease, to provide insights into the pathogenesis of the disease.

Results

Salivary Gland Gene Expression Differentiates Most Patients with pSS from Controls. Gene expression of minor salivary glands was compared between 7 patients with pSS and 7 controls, with 28 cDNA microarrays. Among the 7,261 genes analyzable, 424 genes showed significant differential expression (215 up-regulated, 209 down-regulated) in patients with pSS compared with controls (Table 3, which is published as supporting information on the PNAS web site). Among the 7,261 genes, 3,408 had no missing values in all 28 microarrays, and 120 of these showed significant differential expression between patients and controls (top 120 differentially expressed genes) (Table 4, which is published as supporting information on the PNAS web site).

Unsupervised clustering of all analyzable genes by use of TREEVIEW resulted in no particular grouping of the samples. Unsupervised hierarchical clustering was then applied to the data set of 120 genes to identify expression patterns, which provided a molecular signature allowing for differentiation of most patients with pSS from healthy controls (Fig. 1).

Differential Expression of Genes Involved in IFN Pathways in Salivary Glands of Patients with pSS. Gene ontology analysis was used to organize the expression data of the 424 differentially expressed genes into their functional relationships on the basis of biological processes. Gene ontology terms of interest for autoimmunity included "immune response" for 10% of genes, "cellular defense response" for 12%, "response to external stimulus" for 7%, "cell death" for 5%, and "organogenesis" for 16% of genes. PATHWAYASSIST software revealed 111 genes to be involved in functional pathways. IFN- α and IFN- γ pathways involved 46 (10.8%) of these

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Abbreviations: SS, Sjögren's syndrome; pSS, primary SS; DC, dendritic cell; pDC, plasmacytoid DC; TLR, Toll-like receptor; SLE, systemic lupus erythematosus.

Data deposition: The microarray data reported in this paper have been deposited in the ArrayExpress database (accession nos. E-MEXP-419 and A-MEXP-248).

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Table 1. Differential expression of genes involved in IFN pathways in patients with pSS

Gene name	Gene symbol	Unigen ID	Gene group/function	Mean ratio pSS patients/controls	P_{corr}	P
Genes with increased expression in pSS						
IFN-induced transmembrane protein 1 (9–27)	Hs.458414	IFITM1	IR	2.52	0.030	0.002
CD6 antigen	Hs.436949	CD6	IR	2.40	0.030	0.002
NACHT, leucine-rich repeat and PYD containing 2	Hs.369279	NALP2	CD	2.32	0.030	0.002
promyelocytic leukaemia	Hs.89633	PML	Protein ubiquitination	2.22	0.030	0.002
INF-induced transmembrane protein 2 (1–8D)	Hs.174195	IFITM2	IR	2.04	0.030	0.002
MHC class I polypeptide-related sequence B	Hs.211580	MICB	IR	1.92	0.030	0.002
IFN-induced transmembrane protein 3 (1–8U)	Hs.374650	IFITM3	IR	1.88	0.030	0.002
IL-4 receptor α -chain isoform a	Hs.513457	IL4R	IL4R	1.87	0.048	0.004
Ets variant gene 5 (ets-related molecule)	Hs.43697	ETV5	Nucleic acid binding	1.87	0.030	0.002
Transporter 2, ATP-binding cassette	Hs.502	TAP2	IR	1.76	0.030	0.002
Spleen tyrosine kinase	Hs.192182	SYK	ISC	1.76	0.030	0.002
β 2-microglobulin	Hs.48516	B2M	IR	1.70	0.048	0.004
Mitogen-activated protein kinase kinase kinase 1	Hs.508461	MAP3K1	Kinase activity	1.65	0.039	0.003
TLR9	Hs.436439	TLR9	IR	1.48	0.048	0.004
Diablo homolog	Hs.169611	DIABLO	CD	1.47	0.030	0.002
EphA4	Hs.244624	EPHA4	ISC	1.37	0.030	0.002
Collagen, type XVI, alpha 1	Hs.26208	COL16A1	Cell adhesion	1.35	0.030	0.002
TLR8	Hs.272410	TLR8	IR	1.33	0.030	0.002
Megakaryocyte-associated tyrosine kinase	Hs.437808	MATK	ISC	1.31	0.030	0.002
PKC- δ	Hs.155342	PRKCD	CD/ISC	1.29	0.030	0.002
Chemokine (C-C motif) ligand 20	Hs.75498	CCL20	IR	1.27	0.030	0.002
Genes with decreased expression in pSS						
Chemokine (C-C motif) ligand 18	Hs.412832	CCL18	IR	0.46	0.030	0.002
Suppressor of cytokine signaling 3	Hs.527973	SOC33	ISC	0.18	0.048	0.004

P_{corr} , corrected P value according to Benjamini and Hochberg's correction; IR, immune response; CD, cell death; ISC, intracellular signaling cascade.

to increased secretion of type I IFN (IFN- α/β) and IL-12, which promotes IFN II (IFN- γ) secretion by T lymphocytes, NK cells and DCs (16). In addition, IFN- α and IL-6 secretion by pDCs results in plasma cell differentiation and antibody secretion. Last, activation of pDCs increases their capacities to present antigens, which remain lower than that of myeloid DCs. Thus, the presence of pDCs in the salivary glands of patients with PSS sheds light on the pathogenesis of the disease.

Why are pDCs present in salivary glands of patients with pSS? First, a defective homeostasis of this cell lineage can be hypothesized. Few data are available regarding the ontogeny of pDC lineage (18). ID3, an inhibitory transcription factor of the basic helix-loop-helix family, inhibits the development of pDCs (19). Interestingly, ID3^{-/-} knockout mice were recently reported to develop an autoimmune disease closely mimicking pSS (20). The

migration of pDCs could also be related to specific chemoattractants, promoting their homing into salivary glands in pSS and into various target organs in other autoimmune diseases, such as the skin [systemic lupus erythematosus (SLE) (21) and psoriasis (22)], synovial fluid [rheumatoid arthritis (23) and spondylarthropathy (24)], muscle [dermatomyositis (25)], and cerebral spinal fluid [multiple sclerosis (26)].

The first suspect for induction of IFN- α secretion by TLR stimulation of pDCs is viral infection (27). Interestingly, viruses such as Epstein-Barr, hepatitis C, retroviruses (human T-cell lymphotropic type I), or enteroviruses have been suggested to play a triggering role in the autoimmune epithelitis of pSS (28–31). However, no firm association between viral infections and pSS has been established. Viral components can be recognized by various TLRs, including TLR3, TLR7, TLR8, and TLR9 (27). We dem-

Fig. 3. Quantitative real-time RT-PCR analysis of expression of IFN-inducible genes in salivary glands and ocular epithelial cells. (A) Quantitative RT-PCR analysis of gene expression of IFN-inducible genes and BCMA in salivary glands. Gene expression of TLR8, TLR9, IFITM1, BAFF, and BCMA was significantly increased, and SOCS3 expression was significantly decreased in patients with pSS ($n = 10$) compared with controls ($n = 10$). (B) RT-PCR analysis of expression of IFN-inducible genes in ocular epithelial cells of patients with pSS ($n = 5$) and controls ($n = 8$). BAFF and IFITM1 expression were significantly increased in patients with pSS.

Table 2. Clinical and biological features of patients with pSS

Patients	Age, years	Disease duration, years	Chisholm and Mason's score	Anti-SSA antibody	Anti-SSB antibody	RF	Anti-dsDNA antibody
1	40	4	3	+	+	+	–
2	78	24	4	–	–	–	–
3	80	1	3	+	–	+	–
4	42	5	2	+	+	+	–
5	52	5	3	+	–	–	–
6	46	20	4	–	–	–	–
7	76	1	4	+	+	+	–
8	43	4	4	+	–	+	–
9	55	3	3	–	–	–	–
10	60	2	3	+	+	+	–

RNA from minor salivary gland samples of patients 5, 9, and 10 did not reach a 28S/18S ribosomal RNA ratio between 1.5 and 1.7 and were used only for the quantitative RT-PCR analysis. RF, rheumatoid factor.

inducible genes might be beneficial in pSS, a disease for which no activity marker is currently available. We recently reported the association between increased serum β 2-microglobulin and systemic features in pSS (47). Interestingly, in the present study, the gene expression of β 2-microglobulin, which is also IFN-inducible, was significantly increased in patients with pSS. Thus, the gene expression of some IFN-inducible genes, creating an “IFN score,” might represent a relevant disease activity marker in pSS.

To date, therapy for pSS does not exist. The present results give insights into the lack of efficacy of anti-TNF- α therapy in pSS (48). Indeed, TNF- α -inducible genes were not clustered within the disease molecular signature of the present study. Moreover, because of the tight cross-regulation between TNF- α and IFN-I (49), anti-TNF therapy could lead to further enhancement of IFN pathways, already pathologically activated in pSS. Conversely, the data presented here provide a strong rationale for the development of new therapies to block IFN pathways or to inhibit the consequences of IFN production, such as BAFF secretion, in patients with pSS.

In conclusion, our results support the pathogenic interaction between the innate and adaptive immune system in pSS, a prototypic systemic autoimmune disease. The persistence of the IFN signature might be related to a vicious circle in which the environment interacts with genetic factors (HLA-DR-associated production of autoantibodies and subsequent formation of immune complexes with self-RNA) to drive the stimulation of salivary TLRs.

Materials and Methods

Patients. Ten patients with pSS according to European-American consensus group criteria (50) and 10 control subjects participated in the study after their referral to two reference centers (the departments of rheumatology at the Bicêtre and Strasbourg Hospitals, France). Mean disease duration was 8 years. No features of other autoimmune diseases, including SLE or rheumatoid arthritis was observed in patients. Clinical and immunological features of patients are summarized in Table 2. The control subjects had subjective symptoms of oral or ocular dryness but met none of the objective criteria of SS (no autoantibodies and no lymphocytic infiltrates on minor salivary gland biopsy). Only women were included in both groups. The mean age of patients was 57 ± 17 years (range 40–80). The mean age of the controls was 56 ± 10 years (range 37–70). No subject had ever taken immunosuppressant medication. Informed consent was obtained from all study subjects, and the study was approved by the institutional review board of Bicêtre and Strasbourg Hospitals.

RNA Extraction and Amplification, Synthesis of Fluorescent cDNA from mRNA. Minor labial salivary glands were dissected from the lower lip of subjects. Samples used for the microarray study were immediately placed in RNAlater (Qiagen, Courtaboeuf, France) for

RNA extraction. Samples used for diagnostic focus scoring were fixed in formalin.

Tissue samples were homogenized with a Mixer Mill MM 300 (Qiagen), and total RNA was extracted with an RNEasy MicroKit (Qiagen) according to the manufacturer's recommendations. The quality of RNA was measured with use of a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA): 14 of the 20 samples (seven patients and seven controls) reached a 28S/18S ribosomal RNA ratio of 1.7 or greater and were kept for the following steps of the microarray procedure.

Histologically normal parotid tissue was obtained from four subjects without features of autoimmunity. These tissues were homogenized and pooled to constitute the reference RNA.

One microgram of RNA from the samples and the reference sample was amplified with use of a linear amplification kit (MessageAmp aRNA; Ambion, Huntingdon, U.K.) according to the manufacturer's instructions. The quality and yield of cRNA was analyzed with use of an Agilent Technologies 2100 Bioanalyzer. A total of 250 ng of cRNA from each sample was used for cDNA synthesis. Fluorescent cDNA was synthesized by reverse transcription and indirect labeling and purified from the amplified cRNA with the use of the Fairplay Microarray Labeling kit (Stratagene). Ten microliters of cDNA was separated in two volumes of 5 μ l, one labeled with Cy3 and the other with Cy5 (Amersham Pharmacia) and concentrated by drying under a vacuum in a rotary desiccator in a volume of 4 μ l each.

Hybridization on cDNA Microarrays and Image Acquisition. Gene expression analysis involved the use of cDNA microarrays (Commissariat à l'Energie Atomique, Paris) containing 10,752 human probes, including 1,560 controls, as reported in ref. 51. Twenty-eight microarrays were hybridized according to the manufacturer's recommendations (Genopole, Evry, France) and as reported in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. A dye-swap design was used for Cy3 and Cy5 labeling. For details on image acquisition, see *Supporting Materials and Methods*.

Statistical Analysis of Microarrays. Spots flagged by Standard global locally weighed scatter plots with smoothing (lowess) and normalization (A versus M plot) were then analyzed by using GENESPRING software (Agilent Technologies). Genes with >30% missing values in the patient or control groups were then removed. Analyses involved two sets of data comparing first the probes present in every slide (no missing values) and then the probes for which <30% of values were missing (and were estimated by the LSImpute adaptive method) (52). Statistical analysis involved use of bootstrap resampling with Benjamini and Hochberg's correction.

Unsupervised clustering by sample and gene dimensions by hierarchical clustering was based on the nonparametric Spear-

man correlation distance and complete linkage-group aggregation technique. Results were validated by comparing the results of GENESPRING (Agilent Technologies), CLUSTER/TREEVIEW (53) and HCLUST of R. All of the microarray procedure was MIAME (Minimum Information About a Microarray Experiment) compliant. The microarray data were deposited in the ArrayExpress database (accession nos. E-MEXP-419 and A-MEXP-248).

Gene Ontology Analysis and Analysis of Pathways. Gene ontology analysis involved use of FATIGO web-based software (54). The PATHWAYASSIST software (Stratagene) was used to investigate the functional pathways represented by differentially expressed genes.

Quantitative RT-PCR. The cDNA was amplified by quantitative real-time PCR by using the LightCycler system (Roche Diagnostics) according to the manufacturer's instructions. For details on the conditions of PCRs and on sequences of PCR primers, see *Supporting Materials and Methods*.

Salivary Gland Epithelial Cell Culture. After minor salivary gland biopsy, salivary gland epithelial cells were cultured *in vitro* as described in ref. 55. After 4 weeks of primary culture, IFITM-1 and BAFF gene expression in epithelial cells was assessed at baseline and after 48 h of stimulation with IFN- α or IFN- γ by real-time RT-PCR.

Conjunctival Impression Cytology of the Ocular Surface. Imprints of the bulbar and palpebral conjunctiva were obtained by use of polyether sulfone filter (Supor Gelman Sciences, Ann Arbor, MI) in patients with pSS and controls. RNA was extracted with an RNEasy MicroKit (Qiagen). BAFF and IFITM-1 gene expression was analyzed by quantitative RT-PCR.

Immunohistochemistry. Samples of salivary glands from patients with pSS and controls were immediately frozen and maintained at -80°C until use. Briefly, endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Sections were incubated with mouse monoclonal antibodies against CD123 (IgG₂a, clone 7G3; Becton Dickinson), BDCA2 (IgG₁, clone AC144; Miltenyi Biotec, Paris), and TLR9 (IgG₁, clone 26C93.2; Imgenex, San Diego). Sections were incubated with peroxidase-labeled polymer conjugated to streptavidin (EnVision+; DakoCytomation, Carpinteria, CA). To visualize the bound antibodies, slides were incubated in diaminobenzidine substrate chromogen (DAKO). Tissue sections were counterstained with hematoxylin. In negative control sections, the primary antibodies were omitted, or an irrelevant antibody was applied at the same concentration as the primary antibody.

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