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Activation of the Interferon-α Pathway Identifies a Subgroup of Systemic Lupus Erythematosus Patients With Distinct Serologic Features and Active Disease

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Objective. **Gene-expression studies have demonstrated increased expression of interferon (IFN)–inducible genes (IFIGs) in peripheral blood mononuclear cells (PBMCs) of many patients with systemic lupus erythematosus (SLE), with a predominant effect of type I IFN. This study examined the hypothesis that increased disease severity and activity, as well as distinct autoantibody specificities, characterize SLE patients with activation of the type I IFN pathway.**

Methods. **Freshly isolated PBMCs from 77 SLE patients, 22 disease controls, and 28 healthy donors were subjected to real-time polymerase chain reaction for 3 IFIGs that are preferentially induced by IFNα, and the data were used to derive IFNα scores for all individuals. Expression of IFIGs was significantly higher in SLE patients compared with disease controls or healthy donors. SLE patients with high and low IFNα scores were compared for clinical manifestations of disease, disease severity, disease activity, serologic features, and potential confounders, by bivariate and multivariate analyses.**

Results. **SLE patients with a high IFNα score had a significantly higher prevalence of renal disease, a greater number of American College of Rheumatology criteria for SLE, and a higher Systemic Lupus International Collaborating Clinics damage index (SDI) score than did SLE patients with low IFNα scores. Patients with high scores showed increased disease activity, as measured by lower C3 levels, hemoglobin levels, absolute lymphocyte counts, and albumin levels, and a higher anti–double-stranded DNA (dsDNA) titer, erythrocyte sedimentation rate, and SLE Disease Activity Index 2000 score. The presence of antibodies specific for Ro, U1 RNP, Sm, and dsDNA, but not phospholipids, was significantly associated with a high IFNα score. Logistic regression analysis confirmed that renal disease, higher SDI scores, low complement levels, and presence of anti–RNA binding protein (RBP) autoantibodies were associated with a high IFNα score.**

Conclusion. **Activation of the IFNα pathway defines a subgroup of SLE patients whose condition is characterized by increased disease severity, including renal disease, increased disease activity, reflected in complement activation, and autoreactivity to RBP.**

Systemic lupus erythematosus (SLE) is characterized by extensive defects of the immune system that culminate in the production of autoantibodies reactive with intracellular particles, consisting of nucleic acids and nucleic acid binding proteins. These autoantibodies contribute to the pathologic process underlying the protean clinical manifestations, ranging from skin rash and arthralgias to involvement of major organ systems, and often leading to renal, neurologic, or cardiovascular morbidity. The diversity of serologic and clinical manifestations observed among SLE patients presents important challenges in the diagnosis and management of the disease. In addition, this heterogeneity has hampered the ability of clinical trials to test the efficacy of promising therapeutic agents. Characterization of clinically meaningful subgroups of lupus patients on the basis of

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Table 1. Demographic characteristics of the study subjects*

	SLE patients	Disease controls	Healthy donors	*P*	
				SLE vs. disease controls	SLE vs. healthy donors
No. of subjects	77	22	28	–	–
Mean age (range), years	47 (18–86)	54 (23–86)	35 (20–63)	>0.05	<0.01
No. women	74†	21	21	1.0	0.003
Mean disease duration (range), years	15.2 (1–43)	18.5 (1–34)	NA	0.19	–
Race ratio, no.‡	41:16:8:12	11:2:0:9	16:4:7:1	–	–
Mean prednisone dose (range), mg/day§	6.47 (0–60)	5.3 (0–25)	NA	0.57	–
Median SLEDAI-2K (range)	4 (0–26)	NA	NA	–	–
Median SDI (range)	1 (0–9)	NA	NA	–	–
Mean ESR (range), mm/hour	38.1 (1–148)	45.2 (7–98)	NA	0.37	–
Mean % monocytes (range)¶	24.0 (2.4–64.7)	22.2 (10.2–40.4)	20.0 (8.8–42.4)	>0.05	>0.05
Mean IFNα score (range)	4.26 (0–43)	0.59 (0–5)	0.50 (0–3)	<0.01	<0.01

* SLE = systemic lupus erythematosus; NA = not applicable; SLEDAI-2K = SLE Disease Activity Index 2000; SDI = Systemic Lupus International Collaborating Clinics damage index; ESR = erythrocyte sedimentation rate; IFNα = interferon-α.
† SLE cohort includes 2 men and 1 transgender patient.
‡ Values are the number of whites:African Americans:Asians:Hispanics.
§ All glucocorticoid doses were converted to prednisone equivalent daily doses.
¶ Calculated as the number of monocytes divided by the sum of monocytes and lymphocytes, multiplied by 100.

an immunologic marker would provide an important basis for rational patient selection for clinical trials and could provide new understanding of the relationship between underlying mechanisms of disease pathogenesis and clinical manifestations.

Data from our laboratory and from other studies have focused attention on the role of interferon (IFN) in SLE (1–19). Microarray data have demonstrated elevated expression of a spectrum of genes that are IFN-regulated in the peripheral blood of lupus patients (1–3,16–18). Our recent studies have identified type I IFNs, with IFNα being the prototype, as the predominant trigger of the IFN-induced genes (IFIGs) expressed in SLE. These genes are expressed in a coordinate manner, suggesting that the IFNα pathway is activated globally (5).

The SLE cohort studied in our laboratory comprises patients with a full range of disease activity and severity, with many of these patients being successfully maintained on low doses of corticosteroids or hydroxychloroquine (HCQ) and some with extensive organ involvement requiring immunosuppressive therapy. In collecting data quantifying IFIG expression, we noted that high-level expression of these genes was characteristic of some, but not all, of our patients (5). In light of previous findings suggesting an association of increased disease severity and activity with the IFN signature on microarray (16,17), as well as in vitro data showing a role for RNA- and DNA-containing immune complexes in induction of IFNα (14), we predicted that activation of the IFNα pathway would identify SLE patients with significant organ damage, with active disease, and with distinct autoantibody specificities.

In a cross-sectional analysis, we collected potentially important serologic and clinical data from 77 SLE patients, along with 22 disease controls and 28 healthy subjects. Our data indicate that increased expression of IFIGs, as measured by quantitative real-time polymerase chain reaction (PCR) with results expressed as an IFNα score, identifies SLE patients with a high frequency of renal disease, low serum complement levels, high prevalence of anti–RNA binding protein (RBP) autoantibodies, and perhaps lack of treatment with HCQ. This analysis provides support for further examination of IFIG expression as a potential biomarker of active disease, and suggests a possible pathogenic relationship between induction of autoantibodies to RBP and the production of type I IFN.

PATIENTS AND METHODS

Patients and controls. Seventy-seven SLE patients, 22 disease controls (20 with rheumatoid arthritis [RA] and 2 with inflammatory uveitis), and 28 healthy donors were tested in a cross-sectional and retrospective study design (Table 1). SLE patients and those with RA were followed up at the Hospital for Special Surgery (HSS), and all met the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria for SLE or RA (20,21). Most of the SLE

patients were recruited through the HSS Autoimmune Disease Registry, in 2 ways: 1) patients with relatively quiescent disease (n = 64) were enrolled in a concurrent study of accelerated atherosclerosis in SLE (22) and were recruited consecutively from October 2002 until March 2004; 2) additional patients with apparently active disease were recruited from the HSS arthritis clinic (n = 13) between June 2003 and November 2003. The 2 patients with uveitis were referred from a New York uveitis clinic. All study subjects signed an informed consent form, approved by the HSS Institutional Review Board, which described laboratory investigation of patient material and provided review of concurrent and previous clinical and laboratory data.

Some of the SLE patients and healthy donors were tested on multiple occasions. Comparisons were made among the 3 study groups for IFIG expression, and between the SLE patients with high IFNα scores and those with low IFNα scores, reflecting the relative expression of IFIGs in comparison with that in healthy controls. At the time of the cross-sectional analysis, 4 SLE patients had received pulse glucocorticoid (GC) therapy within the prior 11 days and were not included in the bivariate and multivariate comparisons of clinical and serologic parameters in the SLE subgroups with high or low IFNα scores. Three different SLE patients had additional data available within 24 hours after administration of pulse GC; these latter subjects were considered in the analysis of pulse GC effects on IFIG expression.

Peripheral blood mononuclear cell (PBMC) and plasma samples. All blood was processed immediately after withdrawal, and samples were kept on ice during the procedure. Twenty milliliters of heparinized blood was centrifuged, after which plasma was removed, stored at −70°C, and then fractionated through a Ficoll gradient to obtain PBMCs. These PBMCs were suspended in 10% fetal bovine serum–containing culture medium and distributed in aliquots of 2×10^6 that were then analyzed by flow cytometry or lysed using the RNeasy Mini Kit (Qiagen, Valencia, CA) and stored at −70°C for future RNA isolation. In addition, serum samples were simultaneously obtained from all SLE patients, and aliquots were frozen at −70°C; the remaining sera were tested in the hospital's clinical laboratories for total protein, albumin, blood urea nitrogen, creatinine, C3, C4, and autoantibodies, including anti–double-stranded DNA (dsDNA), anti-Ro, anti-La, anti-Sm, anti–U1 RNP, and anti-cardiolipin (aCL). Finally, complete blood cell counts, the erythrocyte sedimentation rate, urine measures, and lupus anticoagulant (LAC) were determined in the SLE patients.

Flow cytometry. PBMCs were analyzed by FACScan according to forward- and side-scatter parameters. The relative proportions of lymphocytes and monocytes were then assessed by gating on the appropriate populations, and the percentage of monocytes relative to monocytes plus lymphocytes was calculated. On the rare occasion that a significant proportion of neutrophils was present, those cells were not considered in the final calculation of the main 2 cell populations.

Real-time PCR for IFIG expression. To quantify activation of the type I IFN pathway in patient and control cells, genes preferentially induced by IFNα were identified, and messenger RNA (mRNA) encoded by those genes was measured in PBMCs from donors using quantitative real-time PCR. Details of the method used have been described in detail (5,23). Three genes (PRKR, IFIT1, and IFI44) that were predominantly induced by IFNα were identified (5). Expression of these genes was then determined by quantitative real-time PCR in all SLE and control PBMC lysates, and IFNα scores were calculated.

Calculation of the IFNα scores. The mean and SD level of each IFIG in the healthy donor group ($mean_{HD}$ and SD_{HD}, respectively) was used to calculate the expression score of each gene for each study subject, defined as the SD_{HD} value above the $mean_{HD}$ value. Cumulative IFNα scores, representing the sum of the scores for each of the 3 genes preferentially induced by IFNα, were derived for each subject. To implement identification of SLE patients with or without activation of the IFNα pathway, we classified individuals as high IFIG expressers if they fulfilled 1 of the 2 following criteria: 1) overexpression of at least 2 of the 3 IFIGs, with at least 1 gene expressed at a level ≥ 2 SD_{HD} above the $mean_{HD}$, or 2) expression of a single IFIG at a level ≥ 4 SD_{HD} above the $mean_{HD}$. Using these criteria, all healthy donors were distributed in the low IFIG expresser group.

Collection of clinical data. Disease activity and disease-related damage were assessed by each patient's rheumatologist at the time of blood donation, with the use of the SLE Disease Activity Index 2000 (SLEDAI-2K) and the Systemic Lupus International Collaborating Clinics damage index (SDI) (24,25). Current medications were noted by each patient's rheumatologist. These clinical data were verified by one of the authors (KAK) by review of the medical records and incorporation of the laboratory test results in the calculation of the SLEDAI-2K. In addition, all charts were reviewed with emphasis on previous testing for autoantibodies and lupus-specific clinical manifestations. Most SLE patients were tested on more than one occasion for autoantibodies specific for Ro, La, U1 RNP, Sm, dsDNA, aCL, and LAC.

Enzyme-linked immunosorbent assay (ELISA). Donor plasma samples were stored at −70°C until ELISAs were performed. Virgo C-reactive protein (CRP) kits (enzyme immunoassay method, order no. 66203) from Hemagen Diagnostics (Columbia, MD) were used to quantify levels of CRP according to the protocol provided. Upon completion of a sample assay using the kit protocol, absorbance was determined at 450 nm on a Titertek Multiskan Plus microplate reader (Flow Laboratories, McLean, VA).

Statistical analysis. The data distributions of all variables were examined. Two group comparisons of continuous data were assessed using *t*-tests, or the Mann-Whitney test when the data did not have a normal distribution. One-way analysis of variance was used to compare ≥3 groups, with Dunnett's test used to do post-hoc comparisons with a control. Groups of ordinal data were compared using Kruskal-Wallis and Mann-Whitney analyses. Categorical data were compared using Fisher's exact test.

Our primary multivariate analysis consisted of a stepwise logistic regression that was used to identify independent variables that could be associated with a high (designated as 1) or low (designated as 0) IFNα score, based on the available data from 69 (of 73) SLE patients. The variables analyzed were as follows: age, race (proportion of white patients), presence of renal, neurologic, and hematologic manifestations, number of ACR criteria present (modified to exclude renal, neurologic, and hematologic parameters, since these were considered

independently), SDI, proportion of patients with low complement (defined as positive [value of 1] if either one or both of the C3 and C4 levels were below the normal laboratory values, and negative [value of 0] if both C3 and C4 were normal), anti-dsDNA titers, SLEDAI-2K (truncated) (SLEDAI-2K minus the effect of low complement and positive anti-dsDNA antibody titers), hemoglobin levels, presence of anti-RBP or antiphospholipid antibodies, absolute lymphocyte count, prednisone dose, HCQ use, and use of immunosuppressive therapy.

Secondary multivariate analyses were carried out after exclusion of all IFNα scores with a value of 0. The natural logarithms (log) of the IFNα scores were used as the dependent variable in an additional stepwise linear regression analysis for 39 (of 41) patients with complete data, to confirm that a relationship existed independent of the cutoff value used to separate the IFNα high- and low-score groups. In addition, we performed stepwise linear regression for the data on the patients with anti-RBP serologic activity (36 with complete data of a total of 37) since, based on inspection of the raw data, these patients appeared to represent a distinct subgroup of SLE patients with a strong association of high IFNα scores with low complement levels. The log (IFNα score + 1) was used as the dependent variable (we added 1 to allow inclusion of the few 0 values) in this analysis. All regression analyses were performed with the R program (available free of charge at the Web site: www.R-project.org), using Akaike's information criterion to compare the models for their capacity to explain the data (26).

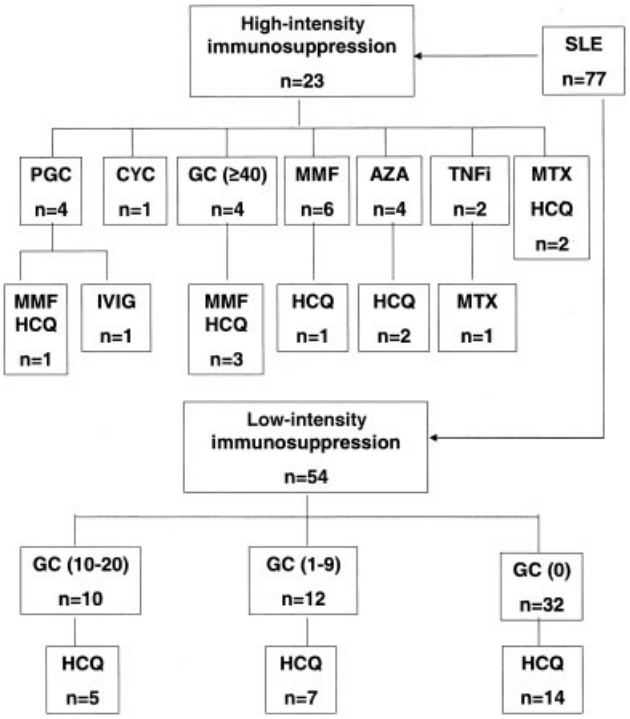

Figure 1. Medical regimens of the patients with systemic lupus erythematosus (SLE) at the time of cross-sectional analysis. The dose of glucocorticoids (GCs) is given in parentheses and is expressed as the equivalent of prednisone in mg daily. Of the 4 patients who received pulse GC (PGC) therapy, 3 were treated 1 day prior and 1 was treated 11 days prior to the study, and these were excluded from the analysis of interferon-α–inducible gene–expression scores (results shown in Table 2). The patient receiving cyclophosphamide (CYC) had received his third intravenous monthly infusion 30 days prior to blood collection. The 2 patients receiving tumor necrosis factor inhibitor (TNFi) were undergoing subcutaneous injections of etanercept at the time of testing. AZA = azathioprine; MMF = mycophenolate mofetil; MTX = methotrexate; IVIG = intravenous gamma globulin; HCQ = hydroxychloroquine.

RESULTS

Increased IFNα gene expression in PBMCs from SLE patients compared with PBMCs from RA patients and healthy donors. Quantitative gene-expression data were collected from 77 SLE patients, 22 disease control patients (20 with RA and 2 with autoimmune uveitis), and 28 healthy donors. An effort was made to match SLE patients to both control groups with regard to sex and race, and the SLE patients and disease controls were well matched for age, disease duration, and daily prednisone dose (Table 1). In general, the SLE patients had low to moderate disease activity, with a median SLEDAI-2K of 4. Only 23 SLE patients received high-intensity immunosuppressive therapy, whereas 70% of the SLE patients were treated with $\leq$20 mg/day prednisone or HCQ (Figure 1).

Quantitative real-time PCR was used to quantify expression of the 3 IFIGs that had been documented to be predominantly induced by IFNα in our previous study (5). PRKR and IFI44 mRNA levels were significantly higher in PBMCs from the SLE patients compared with both control groups, and IFIT1 mRNA levels showed a similar trend (Figure 2). Moreover, the IFNα score, a composite measure of PRKR, IFIT1, and IFI44 mRNA expression relative to their levels in PBMCs from healthy donors, was significantly higher in the SLE group compared with the other groups (Table 1). In fact, only 2 RA patients from the disease control group and none of the healthy donors were classified as being in the IFNα high-score group.

Associations of disease severity, disease activity, and autoantibodies with high IFNα scores in SLE patients. In order to determine whether activation of the type I IFN pathway, as reflected by expression of IFIGs and high IFNα scores, is associated with increased disease severity, increased disease activity, and certain lupus autoantibodies, we compared the high- and low-score SLE groups by bivariate analysis for variables that reflect the above measures, as well as for potential

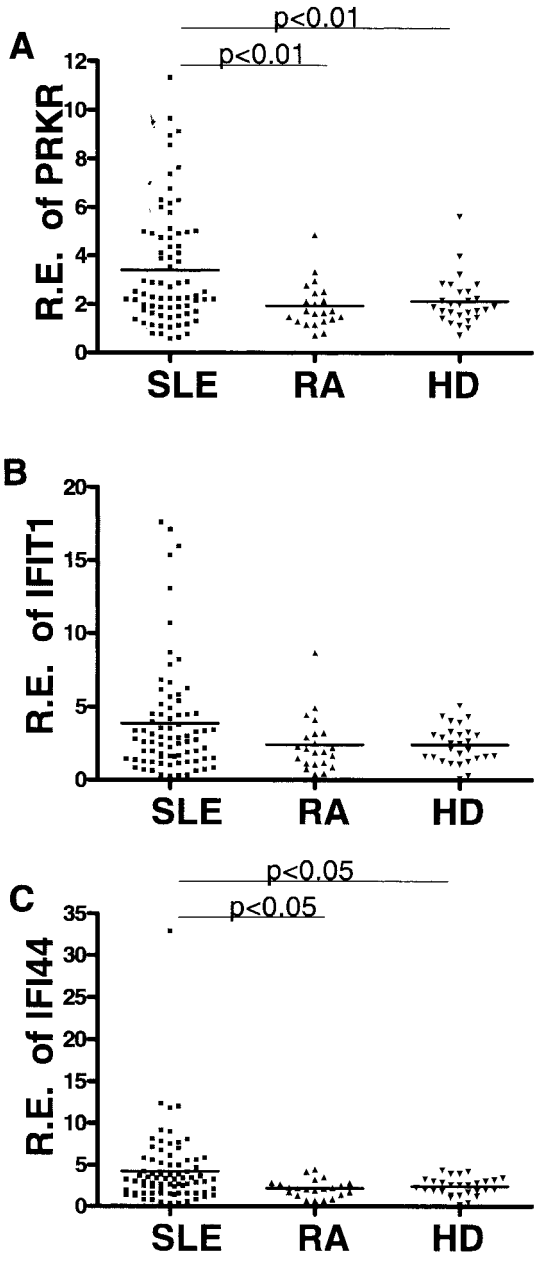

Figure 2. Expression levels of interferon-α–inducible genes (IFIGs) in patients with systemic lupus erythematosus (SLE) and controls. Two million freshly isolated peripheral blood mononuclear cells from SLE patients, disease control patients (with rheumatoid arthritis [RA]), and healthy donor (HD) controls were lysed and used for RNA isolation, reverse transcription, and amplification by quantitative real-time polymerase chain reaction. The relative expression (R.E.) is shown for 3 IFIGs: PRKR (**A**), IFIT1 (**B**), and IFI44 (**C**). Mean values for each group are indicated by the horizontal line.

confounding features. Four of 5 patients had been treated with pulse GCs within the prior 11 days and were therefore excluded from this analysis, because previous studies have suggested a strong inhibitory effect of this therapy on IFIG expression, an observation that has been confirmed by our data (17).

More specifically, comparisons were performed for demographic variables (including age, race, and disease duration), clinical manifestations of SLE, disease extent and severity (as measured by the number of ACR criteria present and the SDI), disease activity and inflammation markers (C3 and C4 levels, anti-dsDNA titers, hemoglobin levels, absolute lymphocyte counts, serum albumin levels, ESR, CRP levels, and the SLEDAI-2K), autoantibody profile (anti-Ro, anti-La, anti-Sm, anti–U1 RNP, anti-dsDNA, and antiphospholipid antibodies, including either aCL or LAC) at the time of assay for IFIGs, and medical therapy (daily prednisone dose, high-dose immunosuppressive therapy [cyclophosphamide, mycophenolate mofetil, azathioprine, methotrexate, intravenous gamma globulin, or tumor necrosis factor inhibitors], and HCQ). All autoantibodies except those against dsDNA were consistently present or absent in a given SLE patient at the time of blood withdrawal and were previously identified on the basis of chart review. The presence of anti-dsDNA autoantibodies was also expressed as present or absent ever in the course of the disease.

As shown in Table 2, whites were less prevalent among the IFNα high-score group, possibly suggesting a genetic contribution to the IFNα score. Among the organ systems that are typically involved in SLE, renal disease was the most prominent in terms of being differentially prevalent among patients with high IFNα scores as compared with those with low IFNα scores; specifically, nephritis or proteinuria was more frequent in the IFNα high-score SLE patients. As seen in Table 2, other manifestations showed a similar tendency but the differences were at a lower significance level. In addition, the extent or severity of disease, as measured by the number of ACR criteria present at the time of IFIG assay and the SDI score, was significantly greater in IFNα high-score compared with IFNα low-score SLE patients.

There is no consensus in the literature regarding the most accurate or useful measure of lupus disease activity. Individual markers, such as serum C3 and C4 levels or the anti-dsDNA antibody titer, or comprehensive activity indices, such as the SLEDAI-2K, have been used in clinical practice or clinical trials to arrive at an accurate assessment of clinically significant immune system activation. In addition to these relatively lupus-specific markers, laboratory or clinical parameters used by physicians to assist in patient management include

Table 2. Comparison of SLE patients with low or high IFNα-induced gene–expression scores*

Variable	No. of patients	IFNα score category		
		High (n = 30)	Low (n = 43)	*P*
IFNα score	73	6 (4.5–14.5)	0 (0–1)	<0.0001
Demographics				
Age, years	73	45.17 ± 14.27	48.42 ± 14.3	0.3419
Race, white	73	10 (33)	30 (70)	0.0038
Disease duration, years	73	14.21 ± 8.02	16.00 ± 10.45	0.4317
ACR criteria/clinical manifestations				
Photosensitivity	69	18 (62)	15 (38)	0.0538
Oral ulcers	69	10 (34)	7 (18)	0.1570
Renal	73	20 (67)	12 (28)	0.0017
Neurologic	73	7 (23)	5 (12)	0.2127
Hematologic	73	22 (73)	22 (51)	0.0882
Lymphadenopathy	73	5 (17)	1 (2)	0.0753
Disease extent/severity				
No. of ACR criteria present	73	6.5 (5–8)	5 (4–6)	0.001
SDI	72	2 (1–3.5)	1 (0–2)	0.0009
Disease activity/inflammation				
SLEDAI-2K score	72	5 (2.5–8)	2 (0–4)	0.0038
C3, mg/dl	73	74.74 ± 26.92	99.61 ± 25.77	0.0002
C4, mg/dl	73	16.24 ± 9.14	19.49 ± 7.61	0.0172†
Low complement‡	73	20 (67)	17 (40)	0.0323
SLEDAI-2K (truncated)	72	2 (0–5.5)	0 (0–4)	0.0621
Anti-dsDNA titers	73	1.5 (0–2.5)	0 (0–1)	0.0093
ESR, mm Hg	66	46.27 ± 30.28	29.28 ± 22.05	0.0105
Absolute lymphocyte count	71	1,207 ± 563	1,575 ± 728	0.0241
% monocytes	63	32.15 ± 13.44	19.67 ± 12.92	0.0005
Hgb	73	11.87 ± 1.56	12.99 ± 1.39	0.0019
Albumin	63	3.85 ± 0.78	4.19 ± 0.5	0.0355
CRP	67	0.24 ± 0.36	0.51 ± 0.83	0.1049
Medical therapy				
Prednisone dose, mg/day	72	10.43 ± 15.94	5.10 ± 10.62	0.0223
Immunosuppressive therapy	73	11 (37)	7 (14)	0.0576
HCQ	73	11 (37)	22 (51)	0.2423
Autoantibody profile				
Anti-Ro	72	21 (70)	10 (23)	0.0001
Anti–U1 RNP	72	12 (40)	4 (9)	0.0035
Anti-Sm	72	7 (23)	2 (5)	0.0289
Anti-RBP	72	25 (83)	12 (28)	<0.0001
Anti-dsDNA	73	20 (67)	16 (37)	0.0178
Anti-dsDNA ever	71	23 (77)	29 (71)	0.7867
aPL	71	8 (27)	15 (35)	0.4472
No. of autoantibodies§	72	2 (1–3)	1 (0–1)	<0.0001

* Values are the mean ± SD, median (25% quartile–75% quartile), or number (%) of patients, depending on whether the data are continuous, ordinal, or dichotomous. ACR = American College of Rheumatology (see ref. 20); dsDNA = double-stranded DNA; Hgb = hemoglobin; CRP = C-reactive protein; HCQ = hydroxychloroquine; RBP = RNA binding protein; aPL = antiphospholipid antibodies (see Table 1 for other definitions).
† The Mann-Whitney test was used, instead of the *t*-test, because the data did not follow a normal distribution.
‡ Presence or absence of low C3 and/or C4 levels.
§ Autoantibodies included anti-dsDNA, anti-Ro, anti-La, anti–U1 RNP, and anti-Sm, with a range of 0–5.

hemoglobin levels, absolute lymphocyte counts, serum albumin levels, and the ESR. We recorded all of these putative measures of lupus disease activity in our patients and found that all were significantly associated with a high IFNα score (Figures 3A–D), except for the CRP level, which, in contrast to the ESR, showed a trend toward increased production in the IFNα low-score group (Table 2). Moreover, in the total patient group, the C3 level was significantly negatively correlated with the IFNα score (Figure 3C). The level of CRP is well known to be less valuable than the ESR as a measure of disease activity in SLE (27,28), and relatively low basal CRP may, in fact, be a characteristic of some lupus patients based on genetic polymorphisms that modulate

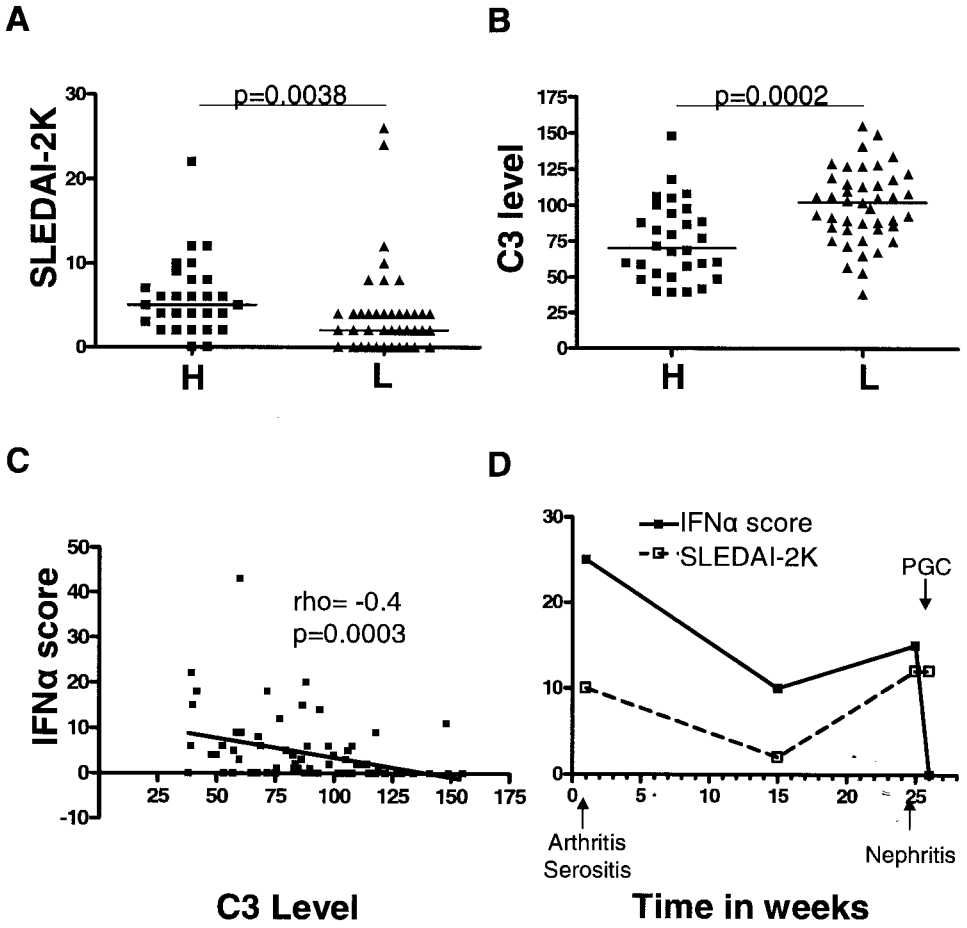

Figure 3. Interferon-α (IFNα)–inducible gene expression and disease activity measures in patients with systemic lupus erythematosus (SLE). SLE Disease Activity Index 2000 (SLEDAI-2K) scores (**A**) and C3 levels (**B**) are depicted for the SLE patients with high (H) and low (L) IFNα scores. Median SLEDAI-2K values and mean C3 values are shown. *P* values are by Mann-Whitney and *t*-tests, respectively. IFNα scores correlate negatively with C3 levels (**C**). The IFNα score parallels disease activity and is rapidly depressed by pulse glucocorticoid (PGC) therapy in a patient with SLE (**D**). The main clinical manifestations accounting for the SLEDAI-2K are given for the 2 time points of high activity. PGC therapy was given as 1 gram of intravenous methylprednisolone 4 days prior to the last time point.

gene transcription or mRNA stability (29). Notably, when the effect of complement and anti-dsDNA antibody was deducted from the SLEDAI-2K, to derive the SLEDAI-2K (truncated) (thus reflecting clinical rather than serologic disease activity), the difference between the 2 groups substantially lost significance. Although we failed to show a significant correlation with the SLEDAI-2K at a group level, the IFNα score paralleled disease activity in 1 SLE patient who was followed up longitudinally (Figure 3D). In summary, the bivariate analysis of several measures of disease activity in our SLE cohort supported an association of increased disease activity with a high IFNα score.

To investigate whether there might be a link between activation of the IFNα pathway and the underlying immunologic processes that result in autoantibody production, we determined the prevalence of autoantibodies specific for dsDNA, RBP, and phospholipids in SLE patients with high and low IFNα scores (Table 2). Anti-Ro autoantibodies had the strongest association with the IFNα high-score group. The other anti-RBP specificities (except anti-La, which was less frequent in high-score patients as compared with low-score patients) were also significantly more prevalent in SLE patients with high IFIG expression, and any one or more of these autoantibodies (anti-Ro, anti-La, anti-Sm, or anti–U1

RNP) was present in 83% of IFNα high-score SLE patients compared with 27% of IFNα low-score patients. Anti-dsDNA autoantibodies present at the time of IFIG assay were associated with a high IFNα score, perhaps reflecting increased disease activity, since consideration of the presence of anti-dsDNA antibodies at other times in the disease course showed no difference between the high- and low-score groups. In contrast to the data regarding anti-RBP autoantibodies, antiphospholipid antibodies did not differ between the 2 groups. These data indicate a striking relationship between autoimmunity targeted at proteins present in RNA-containing RNP complexes and the activation of the type I IFN pathway, perhaps suggesting a mechanistic link between that particular class of self antigens and induction of IFNα.

Interestingly, although the median values for the SLEDAI-2K were consistent with increased disease activity in the IFNα high-score subgroup (Figure 3A), the pattern in 7 of the 43 SLE patients in the IFNα low-score subgroup did not follow this rule, showing a SLEDAI-2K of $\geq$7 as well as relatively low complement levels. Review of these patients revealed that 6 of the 7 had anti-dsDNA autoantibodies, whereas only 2 of the 7 had anti-RBP autoantibodies (of whom 1 patient showed a recent conversion to anti-Ro, and the other had received prolonged, high doses of prednisone, potentially blunting IFIG expression). This analysis suggests that IFIG expression may be most closely associated with disease activity in those SLE patients with anti-RBP antibodies. Indeed, in contrast to the C3 levels in the anti-RBP–positive patients, which were negatively correlated with IFNα scores, C3 levels in SLE patients who did not express that autoantibody specificity showed no significant correlation with the IFNα score (data not shown).

Multivariate analysis. In order to further test the hypothesis that activation of the type I IFN pathway is associated with increased disease severity, increased disease activity, and the presence of anti-RBP autoantibodies, 69 SLE patients with complete data on most of the clinical variables, and particularly those variables suggested by the bivariate analysis, were analyzed using logistic regression, after consideration of possible confounding factors. As shown in Figure 4A, a high IFNα score was highly associated with the presence of anti-RBP, with an increase in the odds ratio by a factor of 12.71. Renal disease, low complement, and the SDI score were also independently associated with a high IFNα score. Although anti-Ro and C3 showed independent associations with a high IFNα score (data not shown), when the variables anti-RBP and low complement were introduced in the analysis (instead of anti-Ro/anti-Sm/anti-RNP and C3/C4, respectively), they resulted in stronger associations with the dependent variable. In addition, white race and treatment with HCQ showed a trend toward a negative association with a high IFNα score, but this did not reach a significance level of less than 0.05. The predictive capacity of the overall model for a high IFNα score is shown in Figure 4B.

In order to test whether the same variables would be identified when the analysis considered the absolute value of the IFNα score, linear regression analysis was performed. The presence of anti-RBP, renal disease, and low complement was again positively correlated with the IFNα score, while HCQ therapy showed a trend toward a negative correlation with IFNα score (data not shown). In addition, to address the prediction that IFNα scores correlate best with disease activity measures in patients who express anti-RBP autoantibody specificities, we performed linear regression analysis on the data from 36 anti-RBP–positive SLE patients whose data were complete for all important variables tested in our previous multivariate analyses. Indeed, the IFNα score was significantly associated with renal disease (P = 0.016) and negatively correlated with C3 levels (P = 0.023), thus confirming the data from the bivariate analysis.

In summary, our logistic regression analysis indicates that the presence of renal disease, low complement levels (a measure of serologic lupus activity), autoantibodies specific for RBP (but not anti-dsDNA or antiphospholipid autoantibodies), and an increase of disease severity, as measured by the SDI, all independently increase the likelihood of having a high IFIG-expression score in the PBMCs of SLE patients. Surprisingly, HCQ therapy, despite the nonsuggestive P value in bivariate analysis, showed a trend toward decreasing the odds ratio for a high IFNα score in multivariate analysis. Linear regression analysis for subgroups of patients with IFNα scores $>$0 and those with anti-RBP serologic activity further supported the importance of renal disease, low complement, and anti-RBP autoantibodies as independent associations with IFN pathway activation.

Association of the proportion of monocytes among PBMCs with high IFNα scores in SLE patients. Our bivariate analysis showed a significant association of a high IFNα score with the percentage of monocytes in the PBMC samples (as measured by flow cytometry). In order to confirm the effect of variable proportions of lymphocytes and monocytes in the PBMC samples on

A

Variable	Beta Coefficient	Odds Ratio (95% C.I.)	P-value
Anti-RBP	2.54	12.71 (2.15, 75.11)	0.004
Renal	2.32	10.21 (1.81, 57.50)	0.007
Low complement (C)	2.00	7.38 (1.39, 39.20)	0.017
SDI	0.41	1.51 (1.01, 2.24)	0.038
HCQ	-1.59	0.20 (0.04, 1.09)	0.058
Caucasian	-1.58	0.21 (0.03, 1.29)	0.085

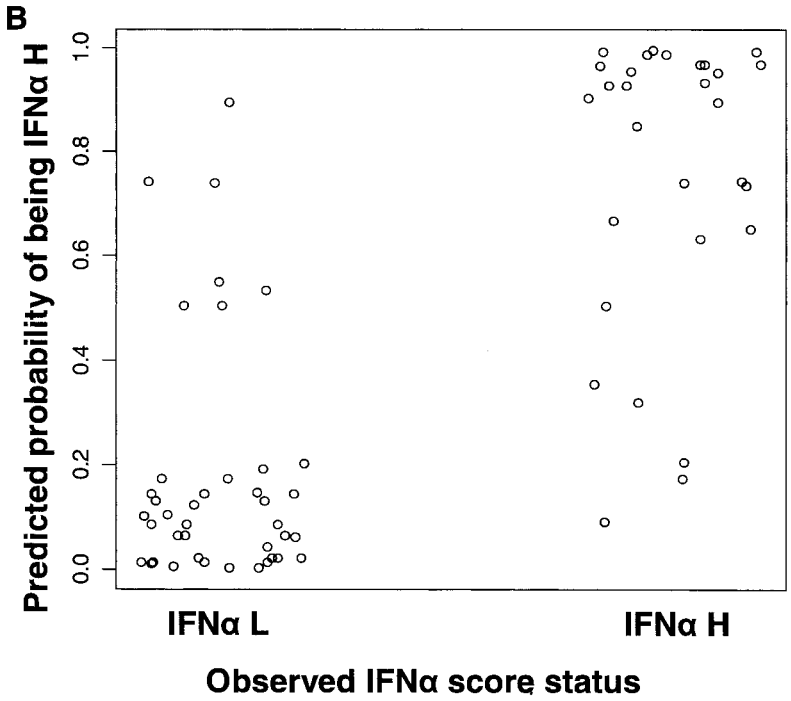

Figure 4. Logistic regression of independently associated variables with interferon-α (IFNα)–inducible gene (IFIG) overexpression in peripheral blood mononuclear cells from systemic lupus erythematosus (SLE) patients. The beta coefficients, odds ratios, and *P* values are shown for the association between each variable and IFIG expression (**A**). Using the 6 independent variables of the model described in **A**, the predicted probabilities of activation of the IFN pathway (having a high IFNα score) in our SLE patients compared with their actual observed IFNα score status (high [H] or low [L]) are graphically displayed (**B**). All observed values were either high or low but were separated out to allow a better depiction. 95% CI = 95% confidence interval; RBP = RNA binding protein; SDI = Systemic Lupus International Collaborating Clinics damage index; HCQ = hydroxychloroquine.

the IFNα score, we repeated the multivariate logistic regression analysis for 55 SLE patients with complete data, assessing the percentage of monocytes as well as the other variables. Indeed, the results were very similar to those from our primary analysis (Figure 4), showing that the percentage of monocytes was also significantly associated with a high IFNα score ($P = 0.009$).

To further examine the effect of the proportion

of lymphocytes relative to monocytes on IFIG expression, we separated the PBMCs from healthy donors into monocyte-enriched and monocyte-depleted fractions, by CD14+ magnetic bead isolation. Baseline expression of PRKR, IFI44, and IFIT1 was 1.6–5.5-fold higher in CD14-depleted, lymphocyte-enriched fractions compared with CD14+ cells. However, when monocyte and lymphocyte populations were stimulated with IFNα, an 8-fold higher induction of IFIT1 was observed in CD14+ cells than in CD14− cells, while the induction of IFI44 and PRKR was 2-fold and 1.5-fold higher, respectively, in that population (data not shown). These data, along with the findings from the logistic regression analysis, indicate that the proposed in vivo activation of SLE monocytes by IFNα (15), as well as the lymphopenia of patients with active SLE, may be factors in the elevated IFIG expression in some patients.

Effects of pulse GCs on IFNα scores. Data on the effects of pulse GCs were available for a total of 7 patients. For 5 of the 7 patients, IFIG-expression data were available for analysis within 1 day after administration of pulse GCs, and for the other 2 patients, data were available by 5 and 11 days after administration of pulse GCs. The IFNα score was 0 in 5 patients, 4 in 1 patient, and 2 in 1 patient, and all of these scores were classified as low according to our definition. In 1 patient for whom IFNα scores were available immediately before and 4 days after treatment with pulse GCs, the IFNα score decreased from 15 to 0 (Figure 3D). Another patient was tested 1 day after, and several months following, pulse GC therapy, and her IFNα score increased from 0 to 12. An additional patient had a score of 0 both before and 1 day after pulse GC therapy. In contrast to the effect of pulse GC therapy on IFIG expression, 3 of 4 patients who received high-dose oral glucocorticoids ($\geq$40 mg prednisone/day) had high IFNα scores (15, 9, and 9) as well as anti-RBP autoantibodies. In summary, pulse GC therapy appeared to rapidly and almost completely down-regulate IFIG expression.

DISCUSSION

In this study we have characterized the clinical and serologic features of SLE patients with high expression levels of IFIGs. Our previous work suggested that in many patients with SLE, the type I IFN pathway is expressed in a coordinate manner, with a number of IFIGs overexpressed in SLE mononuclear cells in relative proportion to the extent to which those same genes can be induced by recombinant IFNα in healthy donor mononuclear cells (5). In the present study, we have used quantitative real-time PCR analysis of 3 IFIGs (PRKR, IFIT1, and IFI44) preferentially induced by IFNα (as compared with IFNγ) to generate an IFNα score, which is reflective of the extent to which the type I IFN pathway is activated in SLE patients compared with healthy donors. In our SLE cohort, representing patients with a wide range of clinical manifestations and levels of disease activity, 41% expressed high levels of IFIGs. In contrast, the mean IFNα score of the disease control patients, comprising predominantly patients with RA, was no different than that of the healthy donor controls.

Designation of SLE patients as either high or low expressers based on the IFNα score permitted the testing of the hypothesis that IFNα pathway activation identifies SLE patients with significant disease severity, with active disease, and with distinct autoantibody specificities. Both bivariate analysis and multivariate logistic regression analysis were used in the total SLE population with complete data. Moreover, secondary multivariate linear regression analysis was performed in those subgroups with IFNα scores $>$1, to confirm that the findings of the primary analysis were true regardless of the cutoff definition of high and low IFNα scores, and in those with anti-RBP autoantibodies, since this subgroup appears to represent a special group of SLE patients with better correlation between IFNα scores and disease activity measures.

Our most striking data, and that which may provide us new clues regarding underlying disease mechanisms, came from analysis of the serologic profiles of the SLE patients. Patients with antibodies specific for RBP, including Ro, Sm, and U1 RNP specificities, were more prevalent in the IFNα high-score group, by bivariate analysis, but anti-La antibodies were not sufficiently frequent to generate significant results. Of these specificities, the initial logistic regression analysis showed a significant association of only anti-Ro autoantibodies with the IFNα high-score group. However, the model was markedly improved when we used anti-RBP as a variable, reflecting the presence of 1 or more of the 4 anti-RBP autoantibodies in the sera. In contrast to anti-RBP, antiphospholipid and anti-dsDNA antibodies were not differentially expressed between the 2 groups, based on multivariate analysis. These data led us to wonder whether the subgroup of SLE patients with anti-RBP autoantibodies might represent a distinct subset of SLE with a common underlying pathogenetic mechanism that involves both autoreactivity to RNA-associated proteins and activation of the type I IFN pathway. Interestingly, the Ro 52-kb autoantigen is both

IFN-inducible and overexpressed in SLE PBMCs (17,30).

Expression of IFIGs was associated with both disease severity and disease activity. The SDI and renal involvement were associated with a high IFNα score in both bivariate and multivariate analyses. Experiments with murine lupus models have implicated both IFNα and IFNγ in nephritis. Notably, SLE renal endothelium typically harbors tubuloreticular inclusions that are thought to be inducible by IFNα (31,32).

Furthermore, expression of IFIGs was associated with a range of clinical and laboratory parameters of increased disease activity on bivariate analysis, including the SLEDAI-2K score. The results of multivariate analysis demonstrated that IFIG activation was best associated with low complement levels among potential markers of disease activity. Our empiric observation that IFIG expression was better associated with disease activity in the anti-RBP–positive SLE patients was confirmed by bivariate and multivariate linear regression assessing the association with C3 levels (negative correlation). The potential for IFIG expression to accurately reflect clinical, and not just serologic, disease activity is supported by longitudinal data from a single patient, but will require extensive additional study. Notably, IFIG expression was not associated with clinical activity as measured by the SLEDAI-2K (truncated) on either bivariate or multivariate analysis, perhaps reflecting the fact that our cohort of SLE patients had relatively inactive disease.

In addition, the more significant negative association between complement levels and IFNα score may reflect the role of immune complexes in both complement activation and IFN production, as demonstrated by the work of Ronnblom and colleagues (9–14). In studies of blood samples from SLE patients, those investigators found IFN-inducing immune complexes that contain antibodies (RNA and/or DNA) and may also include associated RNA or DNA binding proteins (with the nucleic acids and proteins derived from necrotic or late apoptotic cell material). Interestingly, the IFNα-inducing ability of SLE sera was associated with the presence of anti-RBP with or without anti-DNA antibodies, but not with anti-DNA activity alone (14). Recent data from their laboratory and others suggest that several of the Toll-like receptor (TLR) pathways, along with Fc receptors, may mediate uptake of IFN-inducing complexes and initiation of intracellular signaling pathways that result in cell activation and, in at least some cases, IFN production (13,33–35). A more speculative notion is that intracellular complexes, such as RNP particles, or even small RNA, might activate the IFN pathway through intracellular TLR or via TLR-independent mechanisms (36). The triggering of one TLR family member by nucleic acid components of a molecular complex may result in IFN production and antigen-presenting cell activation while concurrently targeting other components of that complex for processing through the antigen-presentation pathways (4). These parallel events would be followed by expression of complex-associated peptides on the activated antigen-presenting cell surface and subsequent T cell activation.

We also observed that an increased proportion of monocytes among SLE PBMCs, as estimated by forward- and side-scatter distribution by flow cytometry, was also associated with the IFNα high-score group, based on univariate and multivariate analyses. Because the 3 IFIGs were, in general, more effectively induced in the CD14+ fraction of healthy donor PBMCs, the association of high monocyte proportion with the IFNα high-score SLE group may indicate increased in vivo activation of these cells by IFNα, as has been described in a recent study (15).

Pulse GC therapy dramatically decreased the level of IFIG expression in all patients who received that treatment, while many patients treated orally with moderate-to-high doses of prednisone had high IFNα scores. Data from others suggest that intravenous pulse GC treatment may decrease the numbers of IFN-producing cells, transiently reducing the stimulus for IFIG expression (37). In contrast to patients treated with pulse GC, IFNα scores were not lower in SLE patients who received immunosuppressive therapies, such as mycophenolate mofetil and azathioprine. Since those therapies are typically administered to patients with quite active disease, the high IFNα scores may reflect high disease activity, or they may reflect a relative lack of effect of the agents on the relevant immunologic pathway. In contrast to the data regarding immunosuppressive agents, patients treated with HCQ showed a trend toward a decrease in the odds ratio for having a high IFNα score. If confirmed, this observation is of considerable interest, since chloroquine (a drug related to HCQ) has been demonstrated to inhibit cell signaling through several of the TLR pathways and thus may also support a role for immune complex–mediated cell activation through TLR pathways in the generation of IFNα (38–40). In vitro studies will be needed to further explore the effects of these therapeutic agents on activation and modulation of the IFN pathway.

Activation of the IFNα pathway could be an important mediator of the immune system alterations

that confer tissue damage in SLE. IFNα has been reported to promote apoptosis through the Fas pathway, potentially leading to increased availability of autoantigens; to induce maturation of antigen-presenting cells, permitting more efficient activation of low avidity self-reactive T cells; to promote Th1 T cell responses; and to inhibit activation-induced T cell death (11,15,41–48). IFNα may also contribute to production of pathogenic autoantibodies by direct and indirect effects on B cells, resulting in differentiation and Ig class switching to IgG and IgA isotypes (49).

The apparent heterogeneity of SLE patients has presented significant challenges to clinicians as well as to investigators who aim to test promising new therapeutic agents for this important autoimmune disease. Our data have defined a subgroup of SLE patients who have more severe disease, with frequent kidney involvement, and more active disease, as measured by complement activation, suggesting that determination of IFIG expression may prove a useful approach to selection of patients for clinical studies. Prospective longitudinal studies are needed to assess the role of IFIG in monitoring disease activity. Finally, the association of IFNα pathway activation and production of autoantibodies reactive with components of RNP particles provides new clues to the underlying mechanisms that drive autoimmunity and production of immune system effectors.

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