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Blood-Borne RNA Correlates with Disease Activity and IFN-Stimulated Gene Expression in Systemic Lupus Erythematosus

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The loss of tolerance and the presence of circulating autoantibodies directed against nuclear Ags is the hallmark of systemic lupus erythematosus (SLE). Many of these Ags are complexed with short, noncoding RNAs, such as U1 and Y1. The amount of U1 and Y1 RNA complexed with SLE patient Abs and immune complexes was measured in a cross-section of 228 SLE patients to evaluate the role of these RNA molecules within the known biochemical framework of SLE. The study revealed that SLE patients had significantly elevated levels of circulating U1 and/or Y1 RNA compared with healthy volunteers. In addition, the blood-borne RNA molecules were correlated with SLE disease activity and increased expression of IFN-inducible genes. To our knowledge, this study provides the first systematic examination of the role of circulating RNA in a large group of SLE patients and provides an important link with IFN dysregulation. ***The Journal of Immunology*****, 2016, 197: 000–000.**

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that is characterized by diverse disease manifestations impacting multiple organs, including the skin, CNS, joints, vasculature, and kidneys (1). A central component of the disease is the presence of autoantibodies directed against a variety of nuclear Ags (2, 3). The normal process of cell death and turnover results in accumulation of circulating nuclear Ags in SLE as a result of impaired clearance of the cellular debris, and this process is thought to be involved in the pathogenesis of the disease (4–6). Autoantibodies complexed with RNA-containing Ags lead to the formation of immune complexes (ICs) that are known to activate immune cell function, disrupt vascular integrity, and cause organ dysfunction (7–10).

Blood cells isolated from SLE patients overexpress many IFN-inducible genes (11–13). The presence of this gene expression signature has led to the widely accepted hypothesis that IFN is overproduced and contributes to the systemic inflammation that is characteristic of SLE. A recent analysis of the SLE patient IFN gene signature suggested that the expression profile is a composite of genes induced by IFN-α, INF-β, and IFN-γ (14).

Type 1 IFNs function as a key part of host innate immunity. Viruses and other pathogens contain elements that are recognized by host cell pattern recognition receptors that, when ligated, trigger IFN production (15). Among these receptors are nucleic acid–sensing TLRs that recognize distinct nucleic acid motifs, with TLR3- and TLR7- recognizing RNA molecules (16, 17). A large body of data from animal studies, as well as human studies, demonstrates the importance of TLR7 in activating IFN production in SLE. For example, in genetic studies of several mouse strains, TLR7 is the sole requirement for accelerated autoimmunity (18, 19). Additionally, human genetic studies in SLE patients similarly revealed the central role of TLR7 and IFN production in the disease (20, 21). These TLRs are localized within endosomal compartments where they are sequestered from host-derived ligands (16, 22). However, host cell nucleic acids can activate nucleic acid–sensing TLRs following internalization into immune system cells (23–27). One candidate ligand, U1 RNA, is a noncoding ubiquitously expressed transcript that functions as a component of the spliceosome, a ribonucleoprotein assembly involved in the processing of pre-mRNAs, and is known to bind to multiple protein Ags recognized by SLE patient autoantibodies (28, 29). When bound to an Ab, the RNA-containing Ag can be internalized via receptor-mediated endocytosis facilitated by Fc, complement, and BCRs (24, 30, 31), resulting in delivery of the U1-RNA to endosomal TLRs and activation of downstream inflammatory pathways. Numerous studies demonstrated the ability of ICs to effectively activate TLRs. For example, mixing SLE patient sera with necrotic or apoptotic cell extracts results in the production of IFN by healthy donor blood cells in an IgG-dependent and

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Abbreviations used in this article: AI, Ab index; HV, healthy volunteer; IC, immune complex; ISM, IFN signature metric; qPCR, quantitative real-time PCR; RA, rheumatoid arthritis; RT-qPCR, reverse-transcription qPCR; SELENA, Safety of Estrogens in Lupus Erythematosus National Assessment; SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Index.

RNase-sensitive manner (32, 33). ICs also activate the NLRP3 inflammasome in human monocytes, leading to production of IL-1β (34) and activation of the oxidative burst response in neutrophils (35, 36).

Assays for detecting circulating ICs were described, but methodologies for quantifying specific RNAs associated with these complexes were not (37–39). In this study, we provide the first direct measurement, to our knowledge, of RNA molecules complexed with SLE patient autoantibodies. The target RNAs, U1 and Y1, were recovered in association with protein A–bound Igs from a broad cross-section of 228 SLE patients. The amount of U1 and Y1 RNA associated with the captured Abs was significantly elevated in SLE compared with healthy volunteers (HVs). Elevated levels of Ab-bound RNA correlated with disease activity and the expression of IFN-inducible genes. Therefore, this study establishes a link between these blood-borne RNA molecules and SLE disease manifestations, providing the rationale for a therapeutic approach aimed at reducing extracellular RNA.

Materials and Methods

Patients and controls

A total of 228 subjects fulfilling 4 of the 11 American College of Rheumatology criteria for SLE was enrolled from six clinical sites in the United States. The only requirement for entry into this study was meeting SLE Classification Criteria; there were no exclusion criteria. A seventh site (PlasmaLab International) recruited 20 HVs. The study protocol was reviewed and approved by the Western Institutional Review Board (WIRB Pro. No. 20121576) for all sites except one, which used its own institutional review board. Prior to sample donation, each donor signed an institutional review board–approved informed consent form allowing sample collection. Principal Investigators also completed a case report form that included an assessment of concomitant medications and disease activity (Safety of Estrogens in Lupus Erythematosus National Assessment [SELENA]–SLE Disease Activity Index [SLEDAI]) (40).

Sample collection

A Tempus blood RNA tube (Life Technologies) and a heparin Vacutainer tube were obtained from each subject. Plasma was prepared at each of the six clinical sites shortly after each blood draw by a single centrifugation in a clinical tabletop centrifuge, and the plasma samples were transferred to tubes containing recombinant RNase inhibitor, resulting in a final concentration of 1000 U/ml (RNasin Plus; Promega). RNase inhibition is crucial to prevent degradation of plasma RNA by blood-borne RNase activity during processing (41). The resulting plasma and Tempus tube samples were stored at −80 and −20°C, respectively.

Ig RNA analysis

Protein A Plus Agarose (Thermo Scientific Pierce) was used to capture total IgG from plasma. For each sample, three replicate Protein A and a control capture (performed using unconjugated agarose beads) were conducted in parallel. One-milliliter aliquots of each thawed plasma sample were centrifuged at 16,000 × *g* for 5 min at 4°C. A total of 200 μl of the resulting supernatants was transferred into each of three 15-ml tubes containing 4.4 ml of PBS, 0.05% Tween-20, and 400 μl of Protein A Plus Agarose (50% suspension) and a single 15-ml tube containing unconjugated agarose beads in the same buffer. Tubes were incubated for 1 h at 4°C with gentle mixing, after which the mixtures were transferred to disposable columns, and resins were collected. Resins were washed (gravity flow) with three 5-ml volumes of PBS, 0.05% Tween, suspended in 1 ml of PBS, 0.05% Tween, transferred to 1.7-ml microcentrifuge tubes, and collected by brief centrifugation; the resulting supernatants were discarded. RNA was recovered from the washed beads using miRNeasy Micro Kits (QIAGEN), per the manufacturer's instructions. RNA was eluted in 15 μl of RNase-free water and stored at −80°C.

cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kits (Life Technologies); 4 μl of each RNA isolate was reverse transcribed in a 20-μl reaction, according to the manufacturer's instructions. A control reaction lacking reverse-transcriptase was conducted for each plasma sample.

Levels of U1 and Y1 RNA were measured by quantitative real-time PCR (qPCR) using hydrolysis probe-based detection. All primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA). For detection of U1, amplification primers were 5′-CGGAGTGCAATGGATAAG-3′ and 5′-CTGGCAGGGGAGATAC-3′, and the detection probe was 5′-/56-FAM/C-CTGGGAAA/ZEN/ACCACCTTCGTG/3-IowaBlackFQ/-3′. Primer sequences for Y1 were 5′-GGGAAAGAGTAGAACAAGGAGT-3′ and 5′-TGGTCCGA-AGGTAGTGAGTTA-3′, and 5′-/5HEX/TCGATCTGT/ZEN/AACTGACTGT-GAACAATC/3-IowaBlackFQ/-3′ was used as the detection probe.

For qPCR, 2 μl of cDNA was added to 25-μl reactions containing 1× Brilliant Multiplex Master Mix (Agilent Technologies), 30 nM ROX reference dye, 125 nM U1 and Y1 primers, and 62.5 nM U1 and Y1 probes. Incubation conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in an Agilent Technologies MX3005P qPCR instrument. A standard curve containing from 10^1 to 10^7 copies of linearized plasmid DNA containing U1 and Y1 sequences was included in each qPCR run. Ct values were obtained using MxPro software (Agilent Technologies), and standard curves were used to convert Ct values into copy numbers. For each isolate, the background signal detected in cDNA derived from the control bead capture was subtracted from the signal derived from protein A capture. The resulting specific signal was expressed as 1000 copies/ml of input plasma (kcopies/ml). Triplicate U1 and Y1 measurements displayed median coefficients of variation of 18 and 13%, respectively, over the 228 samples analyzed (data not shown).

Blood RNA analysis

RNA isolation, quality control, and Fluidigm Dynamic Array (48 × 48) qPCR analysis were performed by EA Genomics Lab of Q^2 Solutions. The array measured 186 transcripts; 6 of these were included as reference genes for normalization. The remaining 180 genes were selected based on meta-analyses of published and unpublished SLE clinical study gene-expression datasets compiled by the Benaroya Research Institute (13, 42–44). Genes that differentiated healthy individuals from SLE patients or showed a link to disease severity were selected. From this list of modules, representative genes were selected for inclusion in the array. The array was dominated by the presence of known IFN-responsive genes. In addition, genes associated with neutrophil, B cell, and myeloid modules were included. Each RNA isolate was assayed twice, and resulting Ct values were inverted by taking the number of effective cycles in the assay and subtracting the Ct value reported for the gene. As a result, reported Ct values correlate positively with quantity. ΔCt (or normalized) values are corrected relative to the measured set of reference genes. Each target Ct value was normalized to create ΔCt values relative to the average of inverted Ct values of the six reference genes; thus, ΔCt values are also positively correlated with quantity. The IFN signature metric (ISM) score was calculated for each subject as follows: mean Ct value of CMPK2, EPSTI, and HERC5 − mean Ct value of the six reference genes included in the dynamic array (45). This score was multiplied by −1 to give the relative $\log_2$-scaled expression the correct directionality. Subjects with ISM scores > 1.0 were classified as ISM-high.

Serology

Autoantibody analysis was conducted by the Department of Laboratory Medicine at the University of Washington. Anti-dsDNA levels were measured using a commercial ELISA (Bio-Rad), with modifications to the manufacturer's protocol designed to reduce background. A panel of common autoantibodies was measured using Bio-Plex 2200 kits (Bio-Rad). Criteria for scoring a sample positive were based on historical experience testing samples from HVs. For anti-dsDNA, a value > 14 U/ml was considered positive. The following Ab index (AI) values were considered positive: SSA/Ro52 (AI > 1.2), SSA/Ro60 (AI > 1.2), SSB/La (AI > 6.0), Sm (AI > 3.0), SmRNP (AI > 5.0), U1 RNP 68 (AI > 5.0), and U1 RNP A (AI > 5.0). For samples above the normal reference range, absolute AI values were determined following dilution.

Statistical methods

Comparisons of median levels of circulating biomarkers and disease status (HV versus SLE) were assessed with a Mann–Whitney *U* test. The correlations between autoantibodies and autoantibody-bound RNA and SLEDAI score were quantified using Spearman's rank correlation coefficient (ρ), whereas the correlations among autoantibodies and autoantibody-bound RNA were assessed using the Pearson product-moment correlation coefficient. The Fisher exact test was used to test for association between categorical variables.

Results

Patient characteristics

The study enrolled a broad cross-section of SLE subjects with various disease activity and SLE manifestations that was characteristic

of private practice and academic medical center settings. The SLE patient and HV cohorts consisted of 90% females, with average ages of 45 and 41 y, respectively. The average SELENA-SLEDAI score of the SLE cohort was 3.89; 29% of the subjects had a SELENA-SLEDAI score ≥ 6, and 29% had inactive disease, with a SLEDAI score of 0 (Supplemental Table I).

Measurement of RNA

An assay for measuring the amount of RNA associated with Abs was developed to analyze patients with respect to this potential disease mediator. Total plasma IgG was captured with Protein A, including free Abs and ICs. Specific primer/probe sets were designed to allow reverse-transcription qPCR (RT-qPCR) measurement of U1 and Y1 RNA simultaneously. Each of these small noncoding RNAs is known to associate with protein Ags recognized by SLE patient autoantibodies (29, 46).

The quantities of U1 and Y1 RNA recovered in association with Abs isolated from HVs and SLE patients are shown in Fig. 1. Many HVs displayed detectable Ab-bound U1 RNA, and Ab-bound Y1 RNA was detected from all 20 HV samples. RNA measurements from HVs were specific, because levels of U1 or Y1 transcripts recovered from the same plasma samples using Protein A–free agarose beads were 8- and 200-fold less for U1 and Y1, respectively (data not shown). Omission of the reverse-transcription step resulted in greatly attenuated signals, confirming RNA dependence. SLE patients were scored positive if they had U1 or Y1 RNA 2 SDs above the mean level observed in HVs (2200 kcopies/ml [U1] and 17,000 kcopies/ml [Y1]). U1 and Y1 RNA levels were significantly elevated in SLE patients relative to HVs (Fig. 1A, 1B). Levels of U1 RNA were elevated in a greater percentage of SLE patients (59%) than were Y1 RNA levels (29%), and the median difference between SLE and

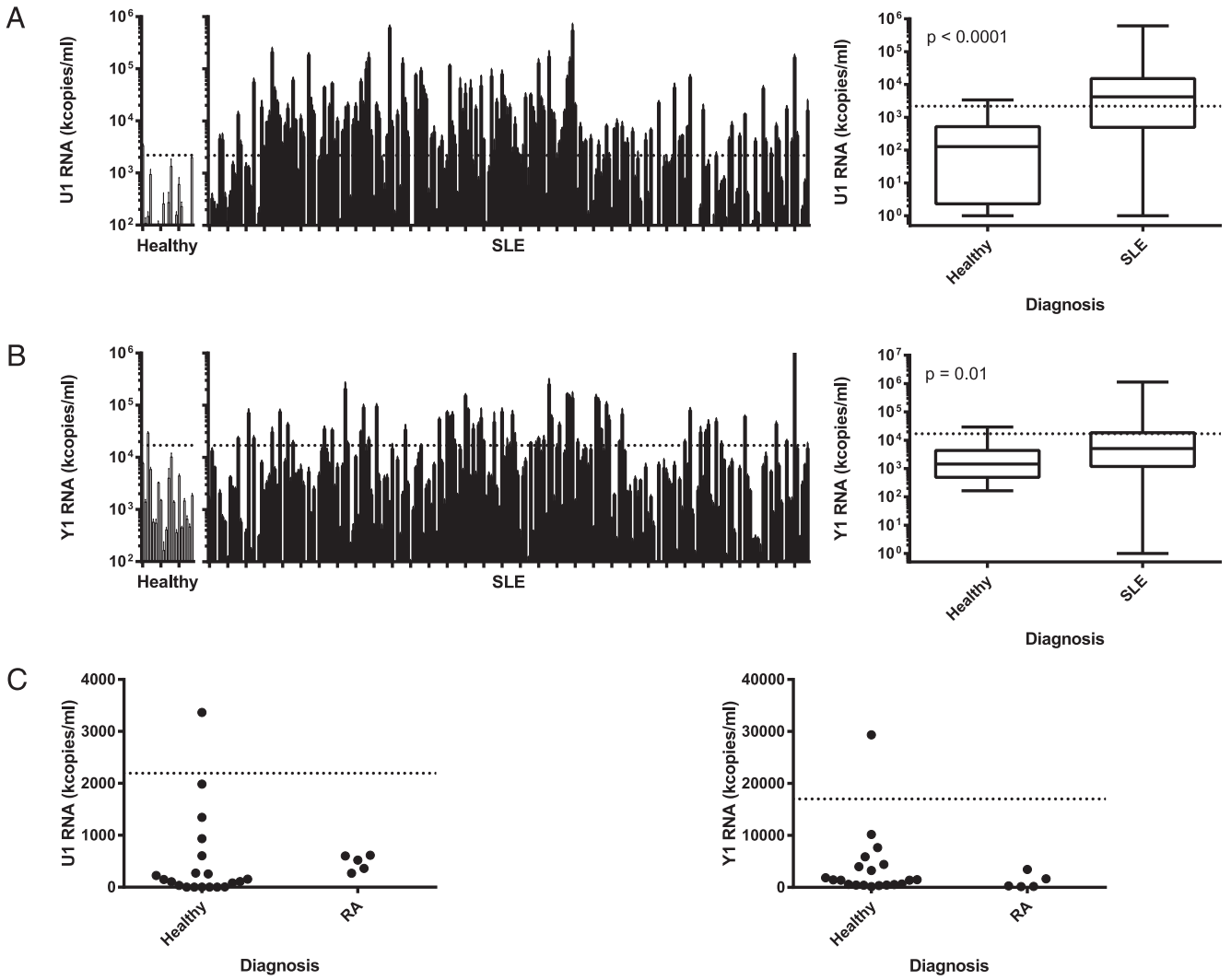

FIGURE 1. Measurement of autoantibody-bound RNA in HVs and autoimmune patients. (**A**) U1 RNA levels measured in HV and SLE patients (kcopies/ml). Each bar represents the mean U1 RNA level of triplicate measurements from a single donor (with error bars showing SD). Dotted lines are placed at 2 SD above mean levels detected in plasma from HVs. The associated box-and-whisker plot compares the distribution of U1 levels in HVs and SLE patients. The midline of each box is placed at the median of all observed values, the box spans from the 25th to 75th percentiles, and the whiskers span the entire range of measured values. (**B**) Y1 RNA levels measured in HV and SLE patients. Each bar represents the mean Y1 RNA level of triplicate measurements from a single donor (with error bars showing SD). Dotted lines are placed at 2 SDs above mean levels detected in plasma from HVs. The associated box-and-whisker plot compares the distribution of Y1 levels in HVs and SLE patients. The midline of each box is placed at the median of all observed values, the box spans from the 25th to 75th percentiles, and the whiskers span the entire range of measured values. (**C**) U1 and Y1 RNA levels recovered from plasma obtained from five RA patients plotted alongside those from the HV cohort. Each dot represents a single donor. For the box and whisker plots, samples for which no Ab-bound RNA was detected were assigned values of 1.0 to enable representation of all values on log-scale *y*-axes. The *p* values were calculated using the two-tailed Mann–Whitney test.

healthy donors was greater for U1 than for Y1 RNA. To determine whether Abs recovered from rheumatoid arthritis (RA) patients were complexed with U1 or Y1 RNA, plasma samples from five patients were subjected to Ab capture and RT-qPCR analysis. Levels of U1 and Y1 RNA recovered from each of the RA patients were comparable to levels observed in HVs (Fig. 1C).

To analyze the daily variability in circulating RNA, a longitudinal substudy was undertaken by obtaining measurements on five consecutive days. The observed fluctuations in autoantibody-bound U1 and Y1 RNA generally varied in a 2–3-fold range over the 5-d period, with subject 4 showing a 1-d 9-fold change (Fig. 2A). The U1 RNA levels remained above the HV threshold during the 5-d study. To assess the day-to-day reproducibility of Ab capture and quantitation from the same sample, repeat measurements of eight plasma samples from the longitudinal study were performed. For all eight samples, the second independent measurements yielded levels of U1 and Y1 RNA in close agreement with the original determination (Fig. 2B). The variability in the repeat measurements averaged 16% for U1 and 13% for Y1 measurements among the group of eight samples.

RNA status, disease activity, and autoantibody status

Examination of the relationship between autoantibody-bound RNA and disease activity demonstrated a statistically significant correlation between each of the two RNAs measured and disease activity, as measured by SLEDAI score (Fig. 3). In contrast, analysis of the seven autoantibodies tested in the study did not show the same strength of correlation with SLEDAI as did the RNA molecules (Supplemental Fig. 1). Of the 228 total SLE subjects in the study, 97 were seronegative for the presence of the tested RNA autoantibodies. Interestingly, despite being negative for the RNA autoantibodies, >40% of these subjects had elevated levels of autoantibody-bound RNA and showed significant associations with IFN gene-expression status and SLEDAI score (Fig. 4).

The panel of autoantibodies assessed in this study included four that bind Ags known to complex with U1 RNA (Sm, SmRNP, U1 RNP 68, U1 RNP A) (29) and three that are known to bind to Y1 RNA (SSA/Ro52, SSA/Ro60, SSB/La) (46). Within this group of

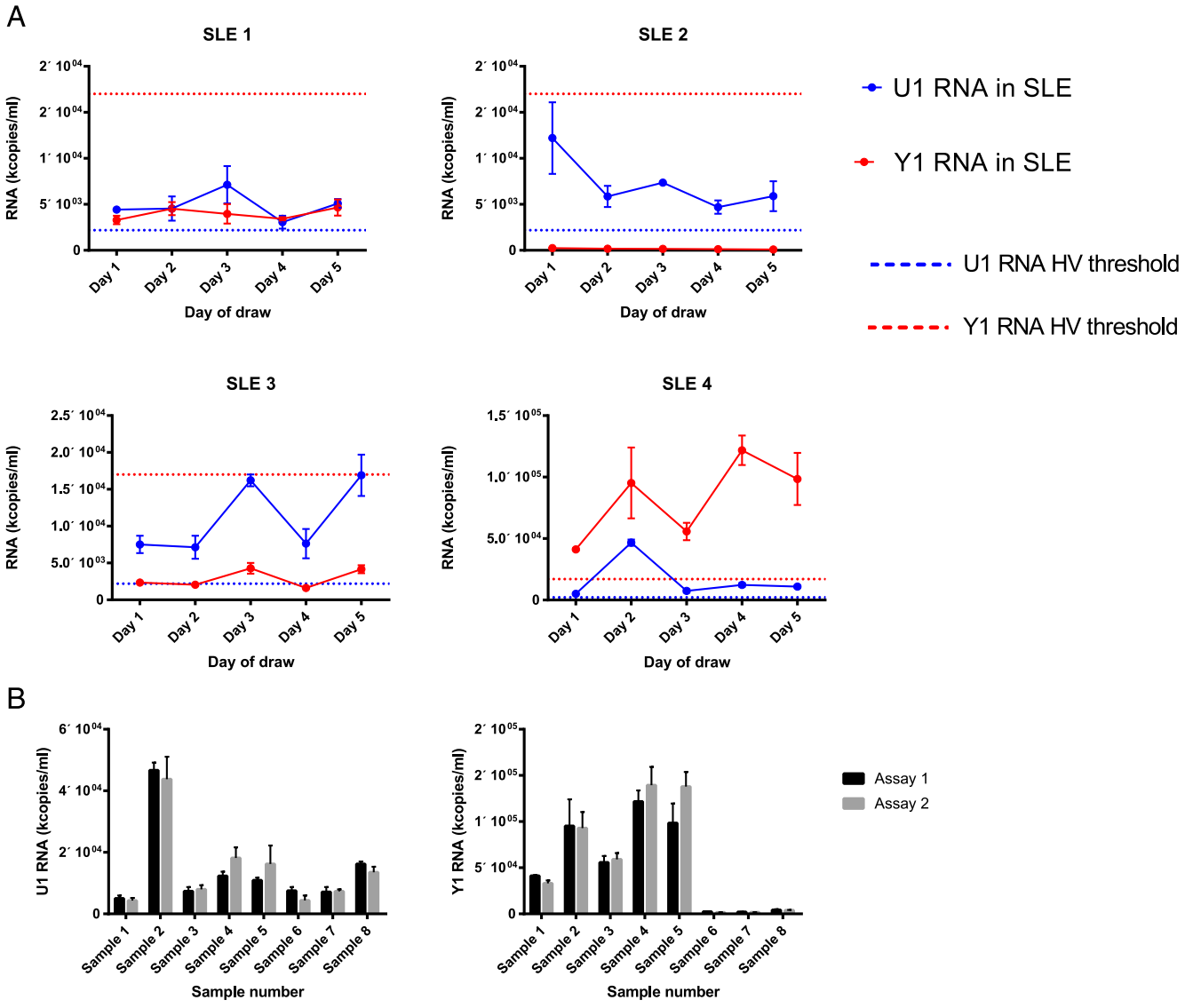

FIGURE 2. Autoantibody-bound RNA levels in four SLE patients over five successive days of sampling. (**A**) Each panel shows U1 and Y1 RNA levels measured over five consecutive days from a single donor. Dotted lines mark 2 SDs above mean levels detected in plasma from HVs. Error bars represent the SD of triplicate measurements. (**B**) Reproducibility of RNA measurements. Results of two independent measurements of U1 (left panel) and Y1 (right panel) RNA in eight plasma samples from the 5-d study. Error bars represent the SD of triplicate measurements.

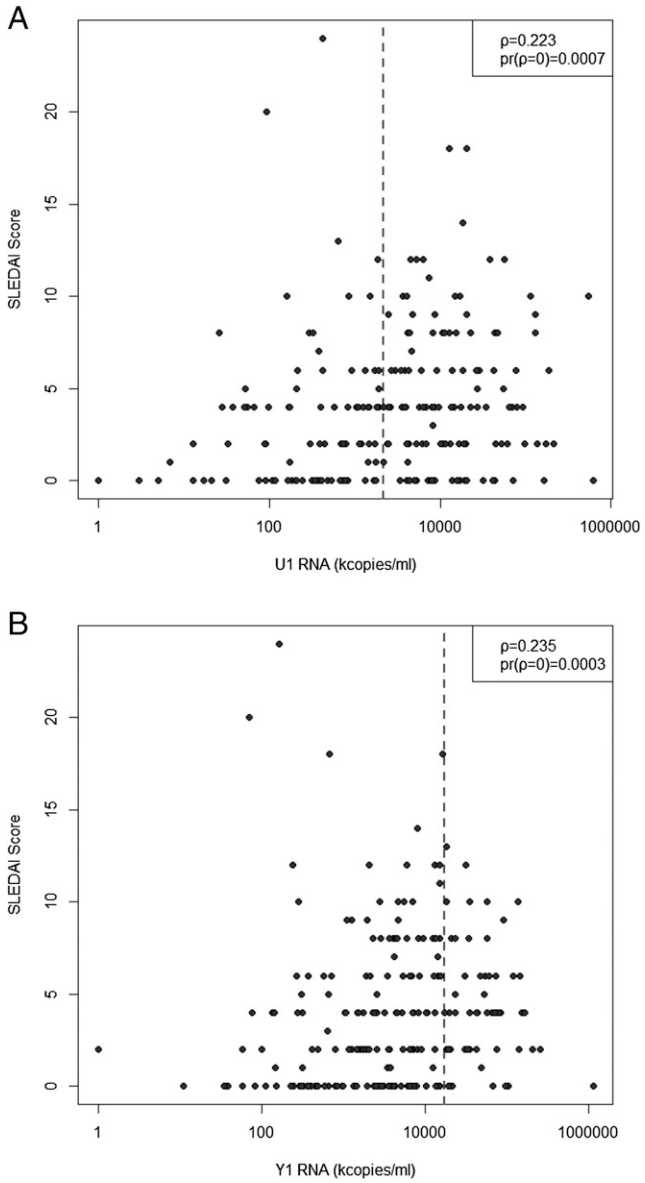

FIGURE 3. Relationship between autoantibody-bound RNA and SLEDAI score. Correlation between U1 RNA and SLEDAI scores (**A**) and between Y1 RNA and SLEDAI scores (**B**) for the 228 subjects in the study. Each symbol marks the value for a single donor. The dashed vertical lines indicate 2 SDs above the mean RNA levels observed in HVs. Correlation coefficients and *p* values were calculated using nonparametric Spearman correlation.

SLE patients, there was a highly significant positive correlation in the quantities of the two RNAs associated with these autoantibodies (Supplemental Fig. 2A, 2B). Anti-SmRNP displayed the strongest associations with Ab-bound U1 RNA (Table I), whereas anti-SSA/Ro60 displayed the strongest associations with Ab-bound Y1 RNA (Table II). However, there was no correlation between autoantibody titer and the corresponding Ab-bound RNA (Supplemental Fig. 2C, 2D).

Blood cell RNA transcript profiling

RNA was prepared from whole blood obtained from each SLE patient and 20 HVs. The expression level of 180 genes thought to be involved in SLE and 6 housekeeping genes were analyzed by qPCR array. The identity and grouping of the genes in the array are shown in Supplemental Table II. A heat map of normalized Ct values for all 228 SLE patients and 20 HVs is shown in Fig. 5. A group of 55

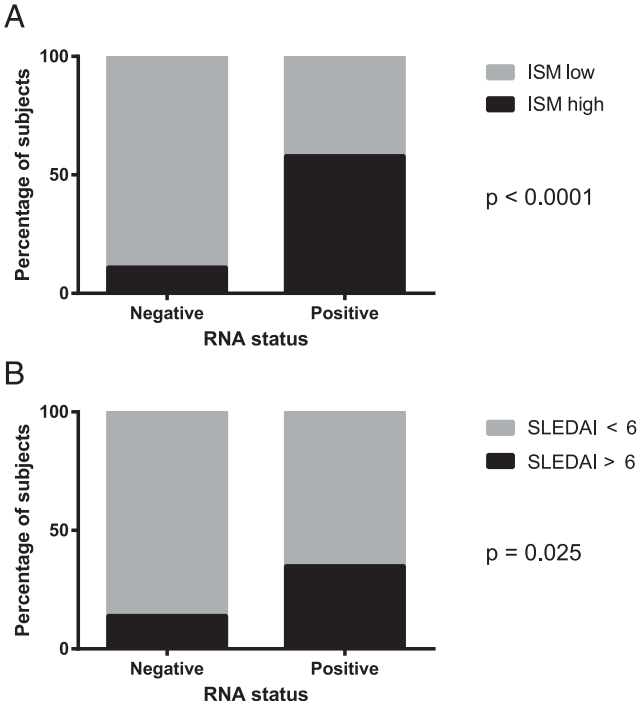

FIGURE 4. Association of IFN status and disease activity with RNA status in a subgroup of autoantibody-negative patients. Of the 228 SLE subjects in the study, 97 were negative for all measured RNA autoantibodies. These 97 SLE patients were divided into two groups, those who were negative for U1 or Y1 RNA ($n = 57$) and those who were positive for U1 or Y1 RNA ($n = 47$), as indicated on the *x*-axis of each graph. (**A**) Percentage of subjects who had low ISM scores (<1) and high ISM scores (>1) are shown within each of these groups of patients. (**B**) The same group of patients was subdivided based on disease activity, using SLEDAI score. The *p* values were calculated from contingency tables using the Fisher exact test.

known IFN-regulated genes clearly separates the subjects into two groups. The low IFN group (green) consists, in large part, of the 20 HVs and those SLE patients lacking autoantibody-bound U1 and Y1 RNA, whereas the high IFN group (red) contains most of the patients positive for autoantibody-bound RNA. In addition, within the IFN high group there are interesting changes to a cluster of genes associated with the neutrophil module that further separates these patients into two fairly distinct subgroups. The subjects' autoantibody-bound RNA status is shown at the bottom of the heat map. The $U1^+$ or $Y1^+$ subjects tend to cluster in the IFN-high group.

Based on calculated ISM scores (45), SLE patients in this study were grouped into IFN signature positive (ISM $\geq$ 1; 64%), and IFN signature negative (ISM $<$ 1; 36%). Elevated levels of autoantibody-bound RNA were significantly associated with the IFN signature positive group (Tables I, II), with U1 RNA showing a stronger association. Also, as noted previously, the association between RNA and ISM status persisted in the subset of SLE patients who were negative for all tested RNA autoantibodies (Fig. 4).

Relationship between RNA and gene expression

To further investigate the relationship between autoantibody-bound RNA and IFN-regulated gene expression, patients were divided into four groups based on RNA status. Group 1 was negative for U1 and Y1 ($U1^-/Y1^-$); group 2 consisted of all patients positive for U1, regardless of Y1 status ($U1^+$); group 3 was $U1^+$ and negative for Y1 ($U1^+/Y1^-$), and group 4 was positive for Y1 and negative

Table I. Characteristics of SLE patients positive and negative for Ig-bound U1 RNA

	Overall (n = 228)	Ig-U1$^-$ (n = 93 [41%])	Ig-U1+ (n = 135 [59%])	p Value
Autoantibody status (n [%])				
dsDNA$^+$	104 (46)	26 (28)	78 (58)	<0.0001
Ro52$^+$	48 (21)	19 (20)	29 (21)	0.8704
Ro60$^+$	87 (38)	28 (30)	50 (44)	0.0518
La$^+$	20 (9)	9 (10)	11 (8)	0.8125
Sm$^+$	38 (17)	9 (10)	29 (21)	0.0194
SmRNP$^+$	68 (30)	11 (12)	57 (42)	<0.0001
U1 RNP 68$^+$	18 (8)	5 (5)	13 (10)	0.3201
U1 RNP A$^+$	40 (18)	8 (9)	32 (24)	0.0042
Gene expression and disease activity				
ISM high (>1; n [%])	147 (64)	35 (38)	112 (83)	<0.0001
SLEDAI (mean ± SD)	3.9 ± 4.1	3.1 ± 4.1	4.4 ± 3.9	

for U1 (U1$^-$/Y1$^+$). Volcano plots were constructed for each of these four groups using the corresponding ΔΔCt values (relative to HVs). Comparison of the volcano plots reveals significant differences in the magnitude of gene expression and associated p values among the four groups of SLE patients (Fig. 6). Most striking were the gene-expression changes and p values in the RNA$^-$ (U1$^-$/Y1$^-$) group compared with the three other groups that were RNA$^+$. All three groups positive for U1 or Y1 RNA displayed similar increases in gene expression. The decreased statistical significance seen in the U1$^-$/Y1$^+$ plot likely reflects the small number of subjects (n = 15) in this group. The top 10 upregulated transcripts observed in the U1$^+$ group were known IFN-regulated genes (12). Within this group, expression change ranged from 46-fold for IFI27 to 7-fold for OAS3. By comparison, the same genes were only modestly upregulated in the U1$^-$/Y1$^-$ group (Fig. 7). This group of genes was also the top 10 upregulated genes in the Y1$^+$/U1$^-$ group and, with the exception of CCL2 replacing OAS3, the top upregulated genes in the U1$^+$/Y1$^-$ group. Relative expression levels of the 180 transcripts measured were highly significantly correlated between the U1$^+$/Y1$^-$ and U1$^-$/Y1$^+$ groups (data not shown).

Discussion

The inflammatory nature of RNA and its role in activation of the innate immune system in autoimmune diseases is widely recognized (7, 23–27). To our knowledge, the present study is the first to systematically quantitate circulating RNA in a large cross-section of SLE patients. Several compelling findings arise from this work. First, plasma from SLE (but not RA) patients demonstrated elevated levels of Ab-bound U1 and/or Y1 RNA relative to plasma from HVs. Thus, increased Ab-bound RNA appears to be a characteristic specific to SLE. Second, the abundance of Ab-bound RNA correlated with disease activity in this cohort of 228 SLE patients. Finally, the presence of autoantibody-bound RNA is highly correlated with the expression of IFN-stimulated genes; conversely patients lacking these RNA molecules demonstrated gene-expression patterns similar to HVs. Taken together with the well-established ability of RNA to stimulate IFN production, the data suggest the involvement of RNA in driving the IFN pathway and disease activity in SLE.

Analysis of the correlation between the autoantibodies tested in this study and SLEDAI scores revealed that there was not a similar degree of correlation between the presence of these autoantibodies and SLEDAI score. The results suggest that RNA complexed with autoantibodies is more closely associated with disease activity and IFN gene stimulation than the autoantibodies themselves. Given the dynamic nature of cellular apoptosis and turnover relative to the largely consistent levels of RNA autoantibodies, Ag production may be the governing moiety modulating inflammatory pathway activation, with autoantibodies serving as delivery vehicles transporting the RNA into proximity with pattern recognition receptors.

Analysis of blood cell RNA expression using a qPCR dynamic array clearly separated SLE patients and HVs based on their IFN gene-expression profile. Among patients demonstrating upregulation of IFN-responsive transcripts, other genes included in the array showed differential expression patterns, including a set of genes associated with neutrophils that appear to subdivide the IFN$^+$ patient group. The presence of RNA complexed with autoantibodies was highly enriched in those patients with elevated expression of IFN-responsive transcripts. Further analysis of the four subgroups of patients positive for U1 and/or Y1 and those negative for these RNA molecules demonstrated a highly statistically significant correlation between the presence of self-antigens containing U1 or Y1 and a group of 10 well-known INF-stimulated genes (Figs. 6, 7).

Table II. Characteristics of SLE patients positive and negative for Ig-bound Y1 RNA

	Overall (n = 228)	Ig-Y1$^-$ (n = 168 [74%])	Ig-Y1$^+$ (n = 60 [26%])	p Value
Autoantibody status (n [%])				
dsDNA$^+$	104 (46)	76 (45)	28 (47)	0.8807
Ro52$^+$	48 (21)	31 (18)	17 (28)	0.1391
Ro60$^+$	87 (38)	51 (30)	36 (60)	<0.0001
La$^+$	20 (9)	12 (7)	8 (13)	0.1824
Sm$^+$	38 (17)	34 (20)	4 (17)	0.0152
SmRNP$^+$	68 (30)	55 (33)	13 (22)	0.1387
U1 RNP 68$^+$	18 (8)	16 (10)	2 (.3)	0.1670
U1 RNP A$^+$	40 (18)	32 (19)	8 (13)	0.4291
Gene expression and disease activity				
ISM-high (>1; n [%])	147 (64)	100 (60)	47 (78)	0.0115
SLEDAI (mean ± SD)	3.9 ± 4.1	3.1 ± 4.1	4.4 ± 3.9	

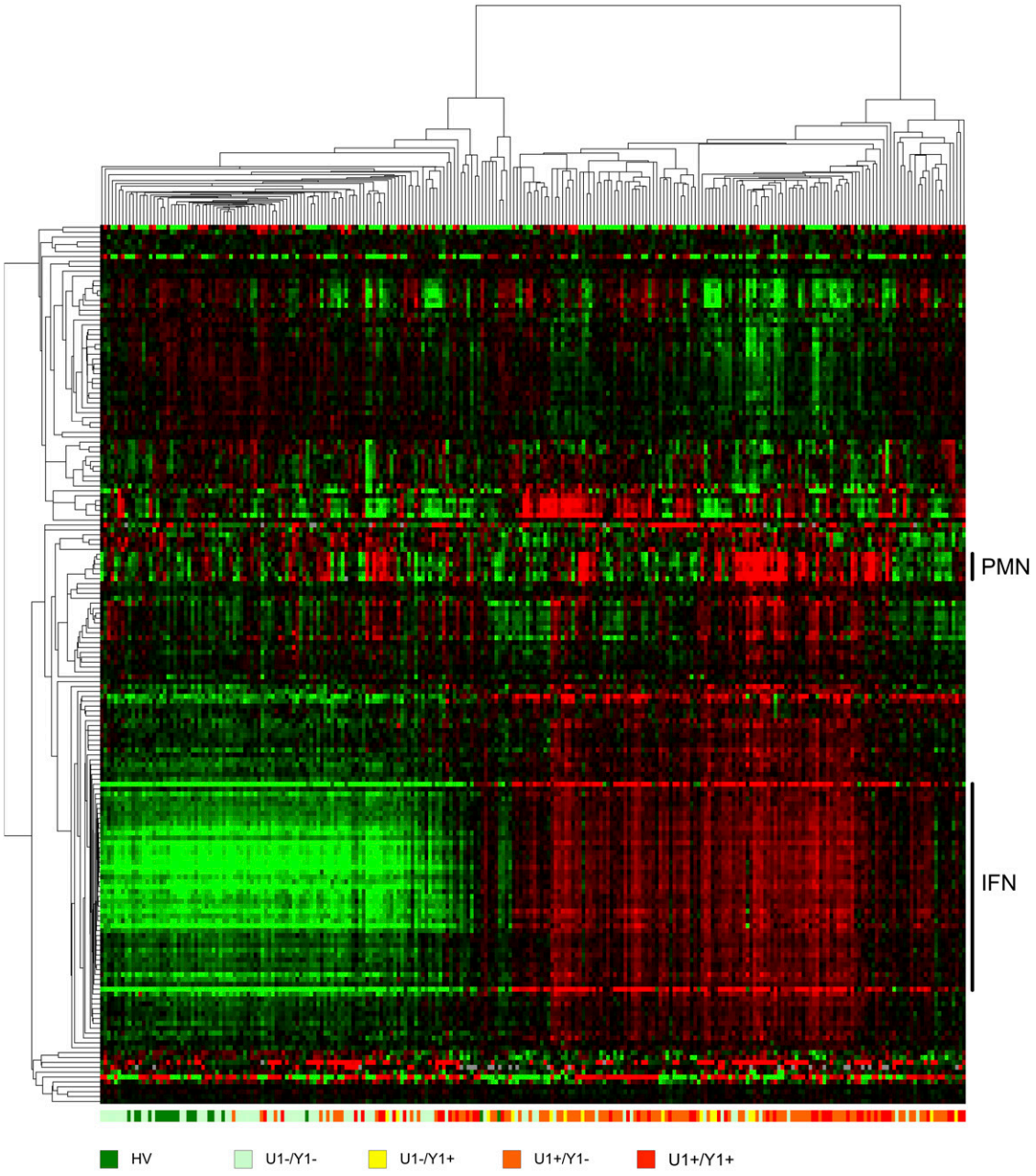

FIGURE 5. Heat map showing expression levels of 180 SLE-associated genes across all study subjects. Blood RNA recovered from HV and SLE donors was analyzed by a Fluidigm Dynamic Array, and normalized Ct values were calculated. Modules of neutrophil genes (PMN) and IFN-regulated genes (IFN) are noted on the ride side of the heat map. The autoantibody-RNA status of each subject is noted along the bottom of the heat map. Data were median centered by gene (red indicates upper or positive values; green indicates lower or negative values after centering). Genes and subjects were hierarchically clustered using Pearson's correlation of the expression as the similarity measure combined with centroid linkage as the method for building the clusters.

U1 and Y1 RNAs were analyzed in the current study based on their known association with protein Ags recognized by SLE patient autoantibodies, but it is likely that Abs may be found in association with other RNA species as well (47). The absence of robust expression of IFN-responsive genes in the $U1^{-}/Y^{-}$ subgroup suggests that U1 and/or Y1 RNA was the primary driver of IFN gene expression in these SLE patients. Some subjects within this subgroup had elevated levels of autoantibodies, so the absence of IFN-inducible gene expression is not simply a reflection of the lack of RNA-binding capacity.

Although highly significant associations were observed between the presence of elevated Ab-bound RNA and the presence of RNA autoantibodies, nearly half of the SLE patients who were positive for Ab-bound U1 RNA were negative for the well-characterized autoantibodies measured in this study. This finding suggests that these subjects may have autoantibodies directed against other Ags that can associate with U1 RNA, such as the U1C protein (48), or autoantibodies that bind directly to U1 RNA (49). Many protein Ags were detected as targets of SLE patient autoantibodies (50).

There was no correlation observed between Ab titers and the level of Ab-bound RNA. This may reflect the vast excess of autoantibody relative to RNA-containing Ag, because autoantibody concentrations in serum are known to be in the μg/ml range (51), and the Ig-bound RNA copy numbers measured in this study are orders of magnitude lower (pg/ml range; data not shown). This finding lends further support to the hypothesis that RNA-containing Ags, rather than the autoantibodies themselves, are the primary determinant in driving inflammation (52).

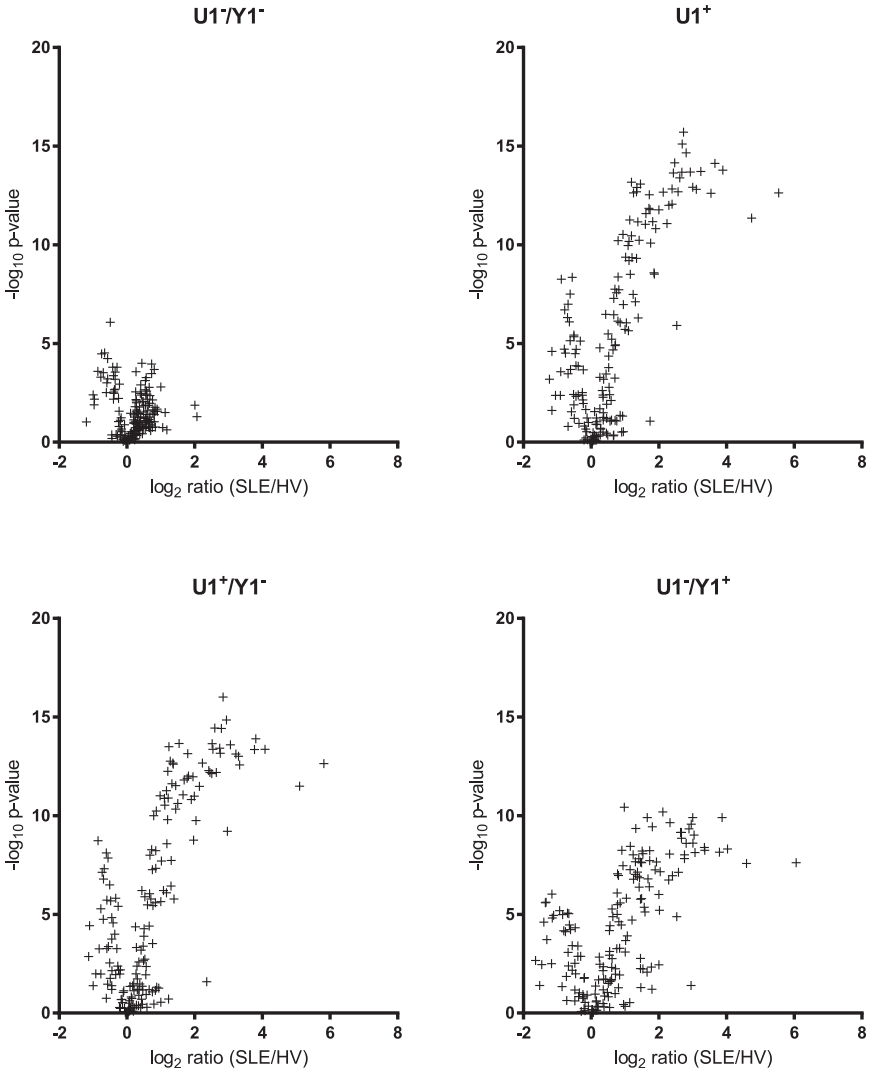

FIGURE 6. Volcano plots showing changes in blood cell gene expression in four groups of patients with different autoantibody-bound RNA status. Each symbol indicates relative expression of a single gene in SLE subjects relative to HVs. The *x*-axis is calibrated in log base 2, and the *y*-axis is calibrated in log base 10. Genes that are more highly expressed by SLE subjects relative to HVs appear as positive values on the *x*-axis, and the statistical significance of the change increases on the *y*-axis.

The presence of autoantibodies directed against RNA-binding proteins was reported to be correlated with increased expression of IFN-inducible genes (9, 41); therefore, it is not surprising that elevated levels of Ab-bound RNA were also associated with an IFN-induced gene-expression signature. However, it was notable that patients negative for all tested RNA autoantibodies still had a strong association between Ab-bound RNA and IFN-stimulated gene expression. Furthermore, there was an association between Ab-bound RNA and elevated SLEDAI scores in these autoantibody-negative patients. Therefore, direct measurement of Ab-bound RNA provides information beyond that available from autoantibody measurements alone.

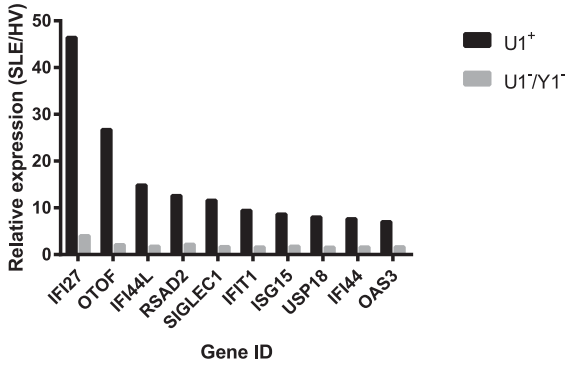

FIGURE 7. Comparison of the relative expression levels of IFN-responsive genes in the $U1^+$ and $U1^-/Y1^-$ subgroups of SLE patients. The 10 genes shown had the greatest fold increase in expression in the $U1^+$ group relative to HVs.

Precisely measuring the amount of Ab-complexed RNA circulating in vivo is a technically challenging undertaking, given that considerable RNase activity is also circulating in the blood (41). Avoiding RNA degradation during sampling necessitates immediate RNase inhibition, as was done in this study. In addition, avoiding the liberation of RNA-containing Ag during plasma preparation is difficult and may lead to the accumulation of additional autoantibodies complexed with RNA Ags that are formed ex vivo during plasma preparation. Notwithstanding the technical limitations noted, it is clear from the correlations observed in this study among patients positive for U1 or Y1 RNA, IFN-stimulated gene expression, and disease activity that Ag-bound RNA molecules are circulating in the blood of these patients and constitute an upstream stimulus driving the production of IFN-α and immune system activation.

SLE flares are known to be triggered by various external stimuli, such as UV light, viral infections, or certain drugs (53, 54). Presumably, the liberation of additional RNA-bound Ag as a result of any of these factors leads to increased Ab-complexed RNA, further exacerbating inflammation. Our longitudinal substudy quantitated daily U1 and Y1 RNA levels over a 5-d period in a subset of four patients to examine fluctuations in these Ab-complexed

RNAs over time. In this analysis, fluctuations of 2–3 fold in Ab-bound RNA measurements were typical. Once an IC forms in the circulation, it is expected to exist transiently (55–57). Steady-state levels of circulating RNA-containing ICs result from the interplay of several biological processes, including release of RNA and associated protein Ags from dying cells, the concentration of autoantibodies in the circulation, the levels of blood-borne RNase, and the rate of clearance of ICs via receptor-mediated processes. Therefore, the steady-state level of RNA-containing ICs is likely to result from the integration of several biological systems and may be quite different among SLE patients.

In this study, low levels of Ab-bound U1 and Y1 RNA were observed in the HVs examined. Given that antinuclear Abs are known to exist in some healthy individuals, this finding is perhaps not unexpected (58, 59). The large amounts of Ab-bound RNA found in many of the SLE patients examined in this study are consistent with the reported defect in the normal clearance mechanism that appears to result in accumulation of cellular debris in the circulation (4–6). Coupled with a loss of self-tolerance and the development of autoantibodies, the presence of circulating RNA provides a proximal trigger of the inflammatory cascade in SLE. The findings presented in this study highlight for the first time, to our knowledge, the connection among circulating RNA, IFN-stimulated gene expression, and SLE disease activity. Therefore, a therapeutic approach aimed at reducing the amount of circulating RNA represents a potentially effective strategy for the treatment of SLE.

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Disclosures

J.A.P., D.J.B., J.R.D., and K.B.E. are consultants to Resolve Therapeutics, LLC, and J.A.P., J.R.D., and K.B.E. own equity in the company. The other authors have no financial conflicts of interest.

References

1. Tsokos, G. C. 2011. Systemic lupus erythematosus. *N. Engl. J. Med.* 365: 2110–2121.
2. Solomon, D. H., A. J. Kavanaugh, and P. H. Schur, American College of Rheumatology Ad Hoc Committee on Immunologic Testing Guidelines. 2002. Evidence-based guidelines for the use of immunologic tests: antinuclear antibody testing. *Arthritis Rheum.* 47: 434–444.
3. Moder, K. G., M. H. Wener, M. H. Weisman, M. L. Ishimori, D. J. Wallace, D. L. Buckeridge, and H. A. Homburger. 2007. Measurement of antinuclear antibodies by multiplex immunoassay: a prospective, multicenter clinical evaluation. *J. Rheumatol.* 34: 978–986.
4. Rose, L. M., D. S. Latchman, and D. A. Isenberg. 1997. Apoptosis in peripheral lymphocytes in systemic lupus erythematosus: a review. *Br. J. Rheumatol.* 36: 158–163.
5. Poon, I. K., C. D. Lucas, A. G. Rossi, and K. S. Ravichandran. 2014. Apoptotic cell clearance: basic biology and therapeutic potential. *Nat. Rev. Immunol.* 14: 166–180.
6. Mahajan, A., M. Herrmann, and L. E. Muñoz. 2016. Clearance deficiency and cell death pathways: a model for the pathogenesis of SLE. *Front. Immunol.* 7: 35.
7. Liu, Z., and A. Davidson. 2012. Taming lupus-a new understanding of pathogenesis is leading to clinical advances. *Nat. Med.* 18: 871–882.
8. To, C. H., and M. Petri. 2005. Is antibody clustering predictive of clinical subsets and damage in systemic lupus erythematosus? *Arthritis Rheum.* 52: 4003–4010.
9. Kirou, K. A., C. Lee, S. George, K. Louca, M. G. Peterson, and M. K. Crow. 2005. Activation of the interferon-α pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. *Arthritis Rheum.* 52: 1491–1503.
10. Li, P. H., W. H. Wong, T. L. Lee, C. S. Lau, T. M. Chan, A. M. Leung, K. L. Tong, N. K. C. Tse, C. C. Mok, S. N. Wong, et al. 2013. Relationship between autoantibody clustering and clinical subsets in SLE: cluster and association analyses in Hong Kong Chinese. *Rheumatology (Oxford)* 52: 337–345.
11. Baechler, E. C., F. M. Batliwalla, G. Karypis, P. M. Gaffney, W. A. Ortmann, K. J. Espe, K. B. Shark, W. J. Grande, K. M. Hughes, V. Kapur, et al. 2003. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc. Natl. Acad. Sci. USA* 100: 2610–2615.
12. Chaussabel, D., C. Quinn, J. Shen, P. Patel, C. Glaser, N. Baldwin, D. Stichweh, D. Blankenship, L. Li, I. Munagala, et al. 2008. A modular analysis framework for blood genomics studies: application to systemic lupus erythematosus. *Immunity* 29: 150–164.
13. Arasappan, D., W. Tong, P. Mummaneni, H. Fang, and S. Amur. 2011. Meta-analysis of microarray data using a pathway-based approach identifies a 37-gene expression signature for systemic lupus erythematosus in human peripheral blood mononuclear cells. *BMC Med.* 9: 65.
14. Chiche, L., N. Jourde-Chiche, E. Whalen, S. Presnell, V. Gersuk, K. Dang, E. Anguiano, C. Quinn, S. Burtey, Y. Berland, et al. 2014. Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type I and type II interferon signatures. *Arthritis Rheumatol.* 66: 1583–1595.
15. Hall, J. C., and A. Rosen. 2010. Type I interferons: crucial participants in disease amplification in autoimmunity. *Nat. Rev. Rheumatol.* 6: 40–49.
16. Theofilopoulos, A. N., R. Gonzalez-Quintial, B. R. Lawson, Y. T. Koh, M. E. Stern, D. H. Kono, B. Beutler, and R. Baccala. 2010. Sensors of the innate immune system: their link to rheumatic diseases. *Nat. Rev. Rheumatol.* 6: 146–156.
17. Barrat, F. J., K. B. Elkon, and K. A. Fitzgerald. 2016. Importance of nucleic acid recognition in inflammation and autoimmunity. *Annu. Rev. Med.* 67: 323–336.
18. Fairhurst, A. M., S. H. Hwang, A. Wang, X. H. Tian, C. Boudreaux, X. J. Zhou, J. Casco, Q. Z. Li, J. E. Connolly, and E. K. Wakeland. 2008. Yaa autoimmune phenotypes are conferred by overexpression of TLR7. *Eur. J. Immunol.* 38: 1971–1978.
19. Deane, J. A., P. Pisitkun, R. S. Barrett, L. Feigenbaum, T. Town, J. W. Ward, R. A. Flavell, and S. Bolland. 2007. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell expansion. *Immunity* 27: 801–810.
20. Lee, Y. H., H. S. Lee, S. J. Choi, J. D. Ji, and G. G. Song. 2012. Associations between TLR polymorphisms and systemic lupus erythematosus: a systematic review and meta-analysis. *Clin. Exp. Rheumatol.* 30: 262–265.
21. Garcia-Romo, G. S., S. Caielli, B. Vega, J. Connolly, F. Allantaz, Z. Xu, M. Punaro, J. Baisch, C. Guiducci, R. L. Coffman, et al. 2011. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci. Transl. Med.* 3: 73ra20.
22. Kono, D. H., M. K. Haraldsson, B. R. Lawson, K. M. Pollard, Y. T. Koh, X. Du, C. N. Arnold, R. Baccala, G. J. Silverman, B. A. Beutler, and A. N. Theofilopoulos. 2009. Endosomal TLR signaling is required for anti-nucleic acid and rheumatoid factor autoantibodies in lupus. *Proc. Natl. Acad. Sci. USA* 106: 12061–12066.
23. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607.
24. Lau, C. M., C. Broughton, A. S. Tabor, S. Akira, R. A. Flavell, M. J. Mamula, S. R. Christensen, M. J. Shlomchik, G. A. Viglianti, I. R. Rifkin, and A. Marshak-Rothstein. 2005. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J. Exp. Med.* 202: 1171–1177.
25. Kelly, K. M., H. Zhuang, D. C. Nacionales, P. O. Scumpia, R. Lyons, J. Akaogi, P. Lee, B. Williams, M. Yamamoto, S. Akira, et al. 2006. "Endogenous adjuvant" activity of the RNA components of lupus autoantigens Sm/RNP and Ro 60. *Arthritis Rheum.* 54: 1557–1567.
26. Lövgren, T., M.-L. Eloranta, B. Kastner, M. Wahren-Herlenius, G. V. Alm, and L. Rönnblom. 2006. Induction of interferon-α by immune complexes or liposomes containing systemic lupus erythematosus autoantigen- and Sjögren's syndrome autoantigen-associated RNA. *Arthritis Rheum.* 54: 1917–1927.
27. Eloranta, M.-L., T. Lövgren, D. Finke, L. Mathsson, J. Rönnelid, B. Kastner, G. V. Alm, and L. Rönnblom. 2009. Regulation of the interferon-α production induced by RNA-containing immune complexes in plasmacytoid dendritic cells. *Arthritis Rheum.* 60: 2418–2427.
28. van der Feltz, C., K. Anthony, A. Brilot, and D. A. Pomeranz Krummel. 2012. Architecture of the spliceosome. *Biochemistry* 51: 3321–3333.
29. Stark, H., P. Dube, R. Lührmann, and B. Kastner. 2001. Arrangement of RNA and proteins in the spliceosomal U1 small nuclear ribonucleoprotein particle. *Nature* 409: 539–542.
30. Means, T. K., E. Latz, F. Hayashi, M. R. Murali, D. T. Golenbock, and A. D. Luster. 2005. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J. Clin. Invest.* 115: 407–417.
31. Erdei, A., A. Isaák, K. Török, N. Sándor, M. Kremlitzka, J. Prechl, and Z. Bajtay. 2009. Expression and role of CR1 and CR2 on B and T lymphocytes under physiological and autoimmune conditions. *Mol. Immunol.* 46: 2767–2773.
32. Båve, U., G. V. Alm, and L. Rönnblom. 2000. The combination of apoptotic U937 cells and lupus IgG is a potent IFN-alpha inducer. *J. Immunol.* 165: 3519–3526.
33. Lövgren, T., M.-L. Eloranta, U. Båve, G. V. Alm, and L. Rönnblom. 2004. Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. *Arthritis Rheum.* 50: 1861–1872.
34. Shin, M. S., Y. Kang, N. Lee, S. H. Kim, K. S. Kang, R. Lazova, and I. Kang. 2012. U1-small nuclear ribonucleoprotein activates the NLRP3 inflammasome in human monocytes. *J. Immunol.* 188: 4769–4775.

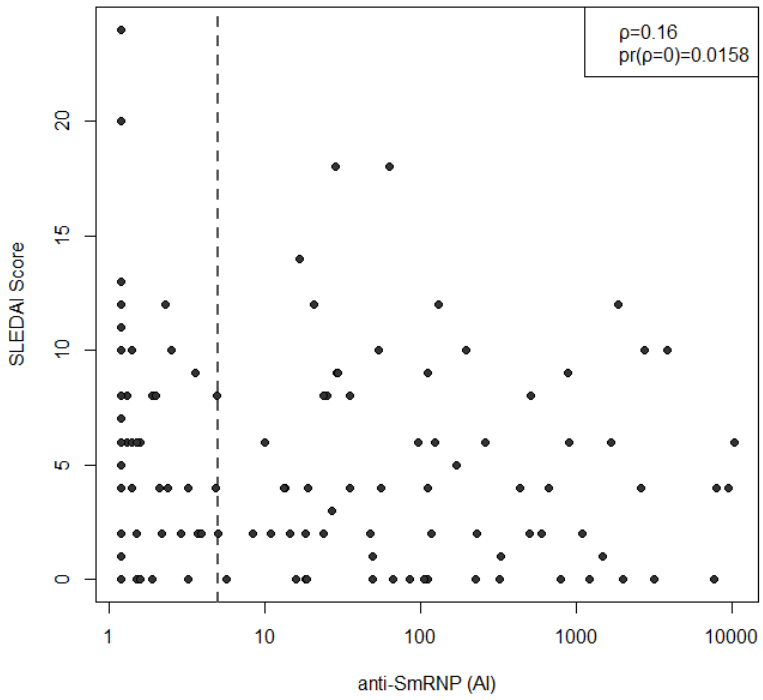
35. Lood, C., M. Allhorn, R. Lood, B. Gullstrand, A. I. Olin, L. Rönnblom, L. Truedsson, M. Collin, and A. A. Bengtsson. 2012. IgG glycan hydrolysis by endoglycosidase S diminishes the proinflammatory properties of immune complexes from patients with systemic lupus erythematosus: a possible new treatment? *Arthritis Rheum.* 64: 2698–2706.
36. Lood, C., L. P. Blanco, M. M. Purmalek, C. Carmona-Rivera, S. S. De Ravin, C. K. Smith, H. L. Malech, J. A. Ledbetter, K. B. Elkon, and M. J. Kaplan. 2016. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat. Med.* 22: 146–153.
37. Theofilopoulos, A. N., C. B. Wilson, and F. J. Dixon. 1976. The Raji cell radioimmune assay for detecting immune complexes in human sera. *J. Clin. Invest.* 57: 169–182.
38. Ahlin, E., L. Mathsson, M. L. Eloranta, T. Jonsdottir, I. Gunnarsson, L. Rönnblom, and J. Rönnelid. 2012. Autoantibodies associated with RNA are more enriched than anti-dsDNA antibodies in circulating immune complexes in SLE. *Lupus* 21: 586–595.
39. Nezlin, R., D. Alarcón-Segovia, and Y. Shoenfeld. 1998. Immunochemical determination of DNA in immune complexes present in the circulation of patients with systemic lupus erythematosus. *J. Autoimmun.* 11: 489–493.
40. Petri, M., J. Buyon, and M. Kim. 1999. Classification and definition of major flares in SLE clinical trials. *Lupus* 8: 685–691.
41. Weickmann, J. L., and D. G. Glitz. 1982. Human ribonucleases. Quantitation of pancreatic-like enzymes in serum, urine, and organ preparations. *J. Biol. Chem.* 257: 8705–8710.
42. Allantaz, F., D. Chaussabel, D. Stichweh, L. Bennett, W. Allman, A. Mejias, M. Ardura, W. Chung, E. Smith, C. Wise, et al. 2007. Blood leukocyte microarrays to diagnose systemic onset juvenile idiopathic arthritis and follow the response to IL-1 blockade. [Published erratum appears in 2009 J. Exp. Med. 206: 2299.] *J. Exp. Med.* 204: 2131–2144.
43. Berry, M. P., C. M. Graham, F. W. McNab, Z. Xu, S. A. Bloch, T. Oni, K. A. Wilkinson, R. Banchereau, J. Skinner, R. J. Wilkinson, et al. 2010. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* 466: 973–977.
44. Park, J., I. Munagala, H. Xu, D. Blankenship, P. Maffucci, D. Chaussabel, J. Banchereau, V. Pascual, and C. Cunningham-Rundles. 2013. Interferon signature in the blood in inflammatory common variable immune deficiency. *PLoS One* 8: e74893.
45. Kennedy, W. P., R. Maciuca, K. Wolslegel, W. Tew, A. R. Abbas, C. Chaivorapol, A. Morimoto, J. M. McBride, P. Brunetta, B. C. Richardson, et al. 2015. Association of the interferon signature metric with serological disease manifestations but not global activity scores in multiple cohorts of patients with SLE. *Lupus Sci. Med.* 2: e000080.
46. Slobbe, R. L., W. Pluk, W. J. van Venrooij, and G. J. Pruijn. 1992. Ro ribonucleoprotein assembly in vitro. Identification of RNA-protein and protein-protein interactions. *J. Mol. Biol.* 227: 361–366.
47. Hung, T., G. A. Pratt, B. Sundararaman, M. J. Townsend, C. Chaivorapol, T. Bhangale, R. R. Graham, W. Ortmann, L. A. Criswell, G. W. Yeo, and T. W. Behrens. 2015. The Ro60 autoantigen binds endogenous retroelements and regulates inflammatory gene expression. *Science* 350: 455–459.
48. Klein Gunnewiek, J. M., L. B. van de Putte, and W. J. van Venrooij. 1997. The U1 snRNP complex: an autoantigen in connective tissue diseases. An update. *Clin. Exp. Rheumatol.* 15: 549–560.
49. Hoet, R. M., P. De Weerd, J. K. Gunnewiek, I. Koornneef, and W. J. Van Venrooij. 1992. Epitope regions on U1 small nuclear RNA recognized by anti-U1RNA-specific autoantibodies. *J. Clin. Invest.* 90: 1753–1762.
50. Sherer, Y., A. Gorstein, M. J. Fritzler, and Y. Shoenfeld. 2004. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Semin. Arthritis Rheum.* 34: 501–537.
51. Maddison, P. J., and M. Reichlin. 1977. Quantitation of precipitating antibodies to certain soluble nuclear antigens in SLE. *Arthritis Rheum.* 20: 819–824.
52. Buyon, J. P., P. Cohen, J. T. Merrill, G. Gilkeson, M. Kaplan, J. James, W. J. McCune, S. Bernatsky, and K. Elkon. 2015. A highlight from the LUPUS 2014 meeting: eight great ideas. *Lupus Sci. Med.* 2: e000087.
53. Hieronymus, T., P. Grötsch, N. Blank, M. Grünke, D. Capraru, T. Geiler, S. Winkler, J. R. Kalden, and H. M. Lorenz. 2000. Chlorpromazine induces apoptosis in activated human lymphoblasts: a mechanism supporting the induction of drug-induced lupus erythematosus? *Arthritis Rheum.* 43: 1994–2004.
54. Achtman, J. C., and V. P. Werth. 2015. Pathophysiology of cutaneous lupus erythematosus. *Arthritis Res. Ther.* 17: 182.
55. Edberg, J. C., G. A. Kujala, and R. P. Taylor. 1987. Rapid immune adherence reactivity of nascent, soluble antibody/DNA immune complexes in the circulation. *J. Immunol.* 139: 1240–1244.
56. Davies, K. A., V. Hird, S. Stewart, G. B. Sivolapenko, P. Jose, A. A. Epenetos, and M. J. Walport. 1990. A study of in vivo immune complex formation and clearance in man. *J. Immunol.* 144: 4613–4620.
57. Davies, K. A., A. M. Peters, H. L. Beynon, and M. J. Walport. 1992. Immune complex processing in patients with systemic lupus erythematosus. In vivo imaging and clearance studies. *J. Clin. Invest.* 90: 2075–2083.
58. Tan, E. M., T. E. Feltkamp, J. S. Smolen, B. Butcher, R. Dawkins, M. J. Fritzler, T. Gordon, J. A. Hardin, J. R. Kalden, R. G. Lahita, et al. 1997. Range of antinuclear antibodies in "healthy" individuals. *Arthritis Rheum.* 40: 1601–1611.
59. Giannouli, E., D. Chatzidimitriou, S. Gerou, E. Gavriilaki, L. Settas, and E. Diza. 2013. Frequency and specificity of antibodies against nuclear and cytoplasmic antigens in healthy individuals by classic and new methods. *Clin. Rheumatol.* 32: 1541–1546.

Supplemental Table 1: Demographics and SLE Medications of study subjects

Attribute	Value
Average Age (years)	45
Average SLEDAI score	3.89
Female	90%
Male	10%
Hispanic/Latino	18%
Caucasian	75%
African American	18%
Asian	5%
Use of following medications:	
Low dose (<10 mg) corticosteroids	38%
High dose (>10 mg) corticosteroids	7.0%
Azathioprine	12%
Mycophenylate mofetil	21%
Methotrexate	8.8%
Hydroxychloroquine	75%
Belimumab	8.3%

Supplemental Table 2 – Identity and module grouping of the RNA transcripts contained in the qPCR gene expression array.

IFN Module (55)									
CCR1	DHX58	GBP1	IFI44	IFIT1	IRF7	MOV10	OAS2	PARP14	TDRD7
CMPK2	EIF2AK2	HERC5	IFI44L	IFIT2	ISG15	MT2A	OAS3	PARP9	TIMM10
CYSLTR1	EPSTI1	IFI27	IFI6	IFIT3	LAMP3	MX1	OASL	PLSCR1	TNFSF13B
DDX60	GALM	IFI35	IFIH1	IFITM3	LY6E	OAS1	OTOF	PRIC285	TOR1B
RNF213	RSAD2	RTP4	SAMD9L	SERPING1	SIGLEC1	SP110	SPATS2L	STAT2	TRIM56
TRIM6	UBE2L6	USP18	XAF1	ZBP1					
B-cell Module (35)									
BACH2	BCAS4	BCL11A	C12ORF57	C15ORF57	CCR7	CD1C	CD79A	CXCR5	CYTH1
EEF1B2	FBL	FCRLA	FOXO1	GPR183	HNRPDL	KIAA1147	LAMA5	LEF1	NAP1L1
OCIAD2	POLR1E	RPL10A	RPL13	RPL14	RPL31	RPS15A	RPS23	RPS25	RPS27
RPS3A	TSPAN13	UBL4A	ZNF22	AFF3					
Neutrophil Module (14)									
ACSL1	ANXA3	B4GALT5	CEACAM6	DEFA4	DUSP1	DYSF	F2RPA	LTF	MMP25
MMP9	MPO	PTGS2	ZNF200						
Myeloid Module (23)									
CTSH	CYFIP1	HLA-DRA	MZB1	TNFRSF1	LILRA3	LILRA5	RNASE2	RERE	IL18
CCL2	EPB41L3	IGJ	RPL27A	CD163	HTATIP2	NFKBIA	UBE2A	IDH1	TNF
BLVRA	FIBP	MAFB							
Erythrocyte Module (12)									
ALAS2	CD34	CLEC4C	GYPB	IFNB1	IL8	MPP1	MT1A	SIAH1	SUGP1
USP8	XPO1								
NK/T-cell Module (10)									
CD160	EOMES	GPR56	GZMB	IFNG	KLRF1	S1PR5	SAMD3	SH2D1B	ZNF683
Poorly Annotated (15)									
ABCA1	ACOT9	ANKRD2	CARD16	CEACAM1	DHRS9	DYNLT	GBP2	IFITM1	IL1RN
IRF9	NEXN	SP100	TNFAIP6	TRIM38					
Poorly Expressing (16)									
ELANE	OLFM4	CDC20	IL37	RPLP1	AVPI1	TRIM29	KRT1	CCL19	CXCL10
IL6	CD133	IL21	IFNA17	CD138	VEGFR2				
Housekeeping (6)				ZNF142	HBP1	DUSP22	NUP37	COQ5	DRAM2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100
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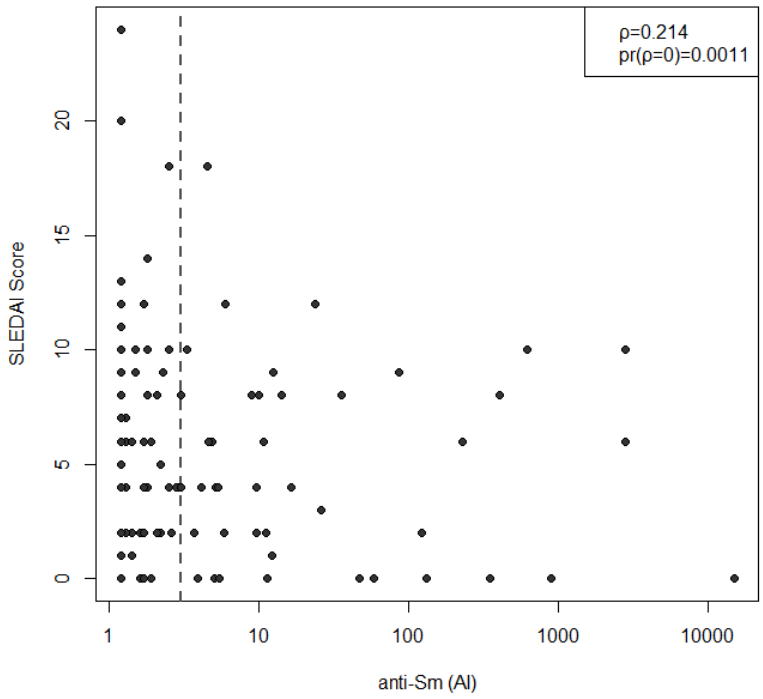
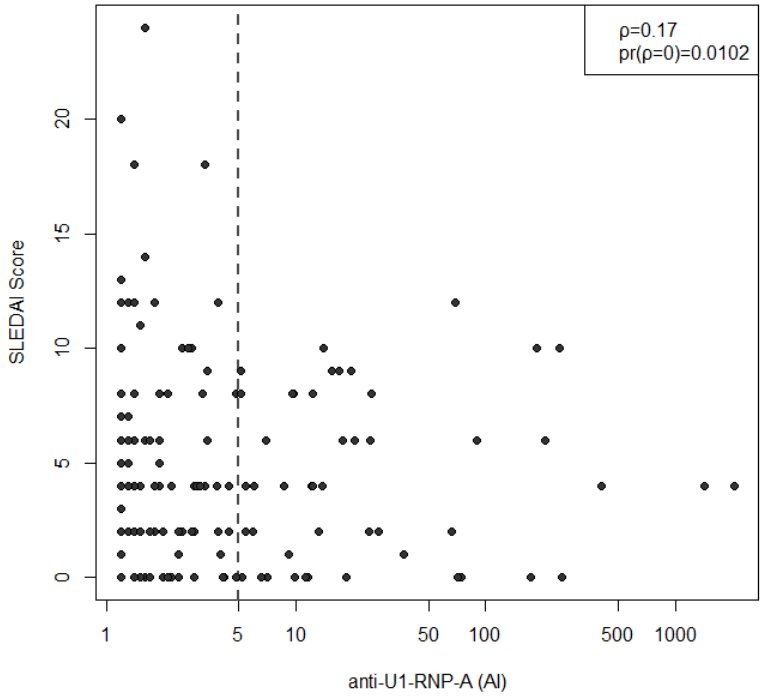
10000
anti-SmRNP (AI)

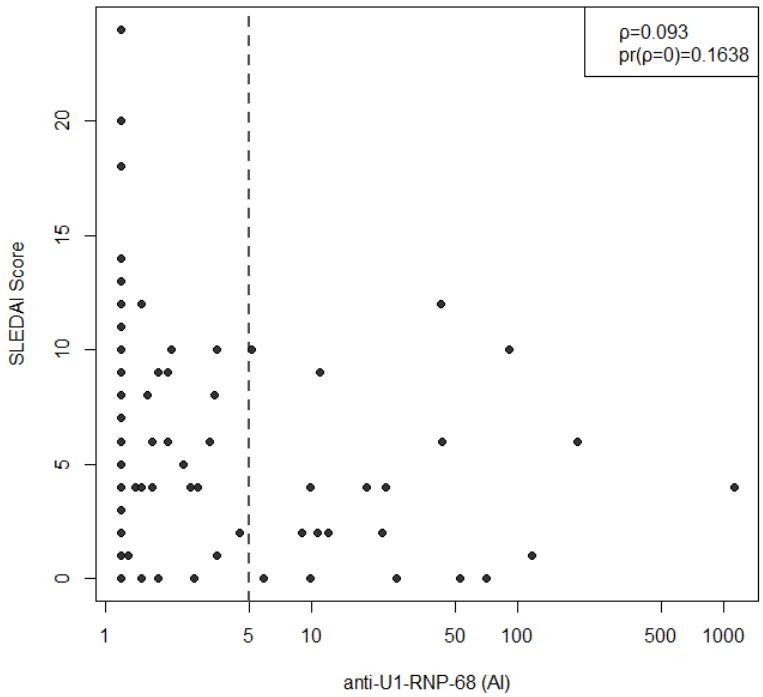
1


ρ=0.214
pr(ρ=0)=0.0011
SLEDAI Score
0
5
10
15
20
1
10
100
1000
10000
anti-Sm (AI)

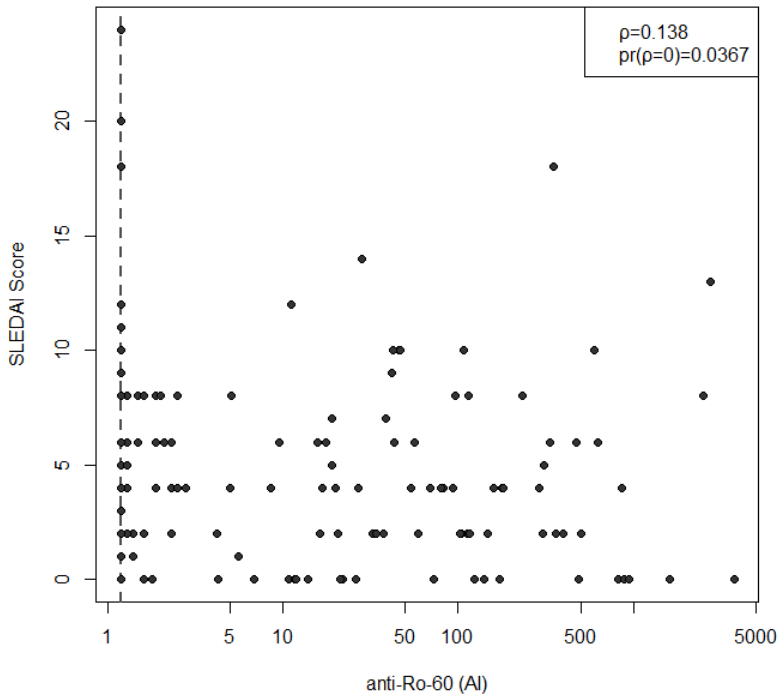
2


ρ=0.17
pr(ρ=0)=0.0102
SLEDAI Score
0
5
10
15
20
1
5
10
50
100
500
1000
anti-U1-RNP-A (AI)

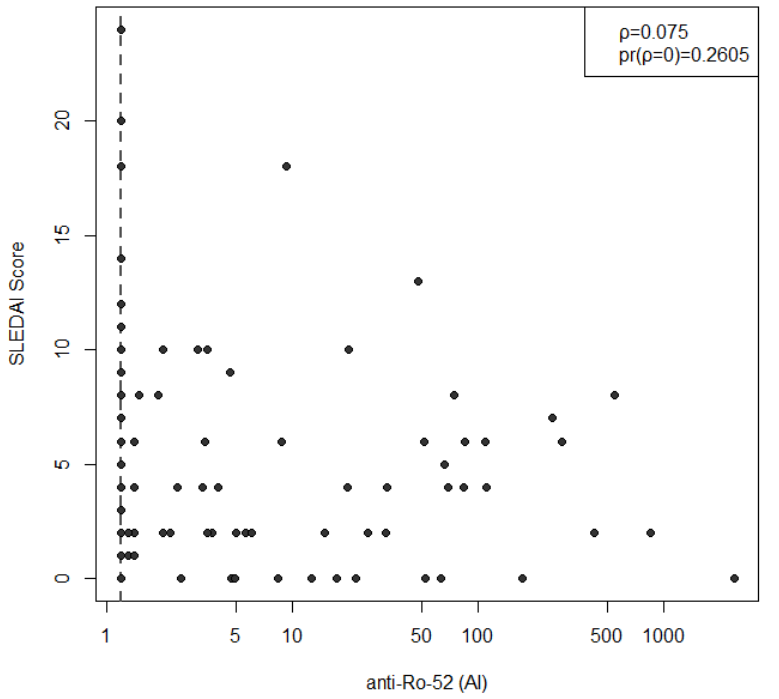
1


ρ=0.093
pr(ρ=0)=0.1638
SLEDAI Score
0
5
10
15
20
1
5
10
50
100
500
1000
anti-U1-RNP-68 (AI)

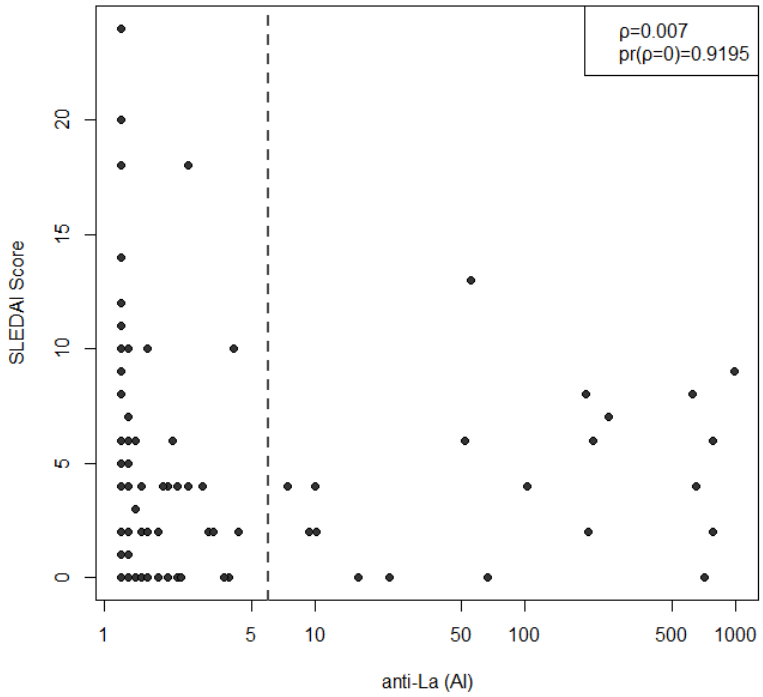
2


ρ=0.138
pr(ρ=0)=0.0367
SLEDAI Score
0
5
10
15
20
1
5
10
50
100
500
5000
anti-Ro-60 (AI)

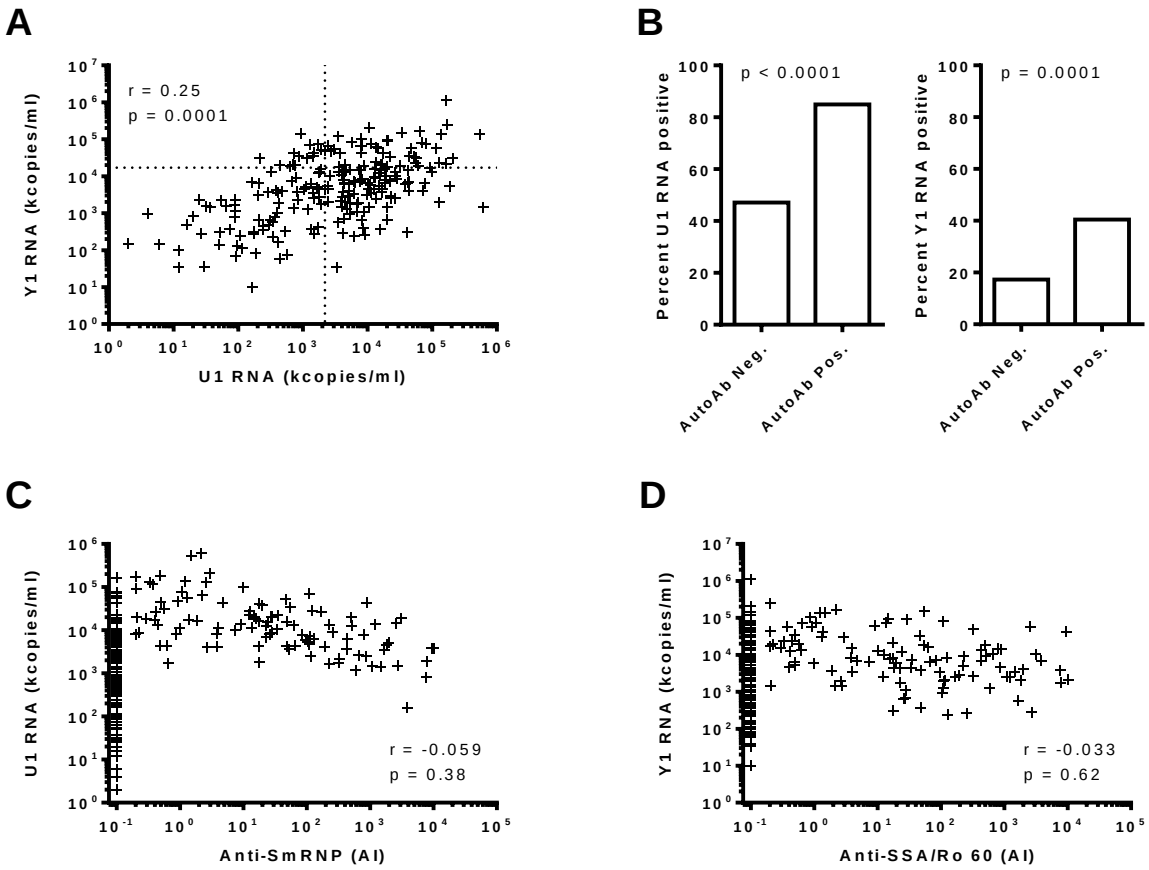
1


ρ=0.075
pr(ρ=0)=0.2605
SLEDAI Score
0
5
10
15
20
1
5
10
50
100
500
1000
anti-Ro-52 (AI)

2



Supplemental Figure 1. Correlation between Autoantibodies and SLEDAI scores. Scatter plots of the seven autoantibodies tested in the study (Sm, SmRNP, U1 RNP 68, U1 RNP A, Ro 52, Ro 60) and SLEDAI score are shown. The dashed vertical line represents the cutoff for positivity. Spearman's rank correlation coefficient (ρ) and the probability that ρ = 0 are provided.



Supplemental Figure 2. Relationships between RNA levels, and autoantibodies. (A) Correlation between U1 and Y1 RNA levels. Each symbol indicates values obtained from a single donor. The correlation coefficient (r) and p-value were calculated using Pearson correlation. (B) Percentage of patients positive for autoantibody-bound RNA based on being positive or negative for the presence of autoantibodies capable of complexing with U1 RNA (left panel) or Y1 RNA (right panel). P-values were calculated from contingency tables using Fisher's exact test. (C) U1 RNA levels plotted against anti-SmRNP titer. (D) Y1 RNA levels plotted against anti-Ro 60 titer. Each symbol indicates values from a single donor. To enable plotting of all values on log-scale axes, autoantibody levels below the limit of detection were set to 0.1 AI. For panels C and D, correlation coefficients (r) and p-values were calculated using Pearson correlation.