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Serum Protein Profiling of Systemic Lupus Erythematosus and Systemic Sclerosis Using Recombinant Antibody Microarrays*[S]

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Systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) are two severe autoimmune connective tissue diseases. The fundamental knowledge about their etiology is limited and the conditions display complex pathogenesis, multifaceted presentations, and unpredictable courses. Despite significant efforts, the lack of fully validated biomarkers enabling diagnosis, classification, and monitoring of disease activity represents significant unmet clinical needs. In this discovery study, we have for the first time used recombinant antibody microarrays for miniaturized, multiplexed serum protein profiling of SLE and SSc, targeting mainly immunoregulatory proteins. The data showed that several candidate SLE-associated multiplexed serum biomarker signatures were delineated, reflecting disease (diagnosis), disease severity (phenotypic subsets), and disease activity. Selected differentially expressed markers were validated using orthogonal assays and a second, independent patient cohort. Further, biomarker signatures differentiating SLE *versus* SSc were demonstrated, and the observed differences increased with severity of SLE. In contrast, the data showed that the serum profiles of SSc *versus* healthy controls were more similar. Hence, we have shown that affinity proteomics could be used to de-convolute crude, nonfractionated serum proteomes, extracting molecular portraits of SLE and SSc, further enhancing our fundamental understanding of these complex autoimmune conditions. ***Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.005033, 1–14, 2011.***

Systemic lupus erythematosus (SLE)[1] (1, 2) and systemic sclerosis (SSc), or scleroderma, (3, 4) are two severe, chronic autoimmune connective tissue diseases with still unknown etiology, complex pathogenesis, heterogeneous presentation, and unpredictable course. As a consequence, the difficulties in diagnosing, classifying, and treating both SLE (1, 5, 6) and SSc (3, 4, 7, 8) are significant. Thus, further studies delineating SLE and SSc, and revealing the underlying disease biology at the molecular level are highly warranted.

SLE is a multifaceted disease, with a prevalence of 40 to 200 cases per 100,000 persons (2), for which the lack of specific biomarkers is critical and impairs the clinical management of these patients (6, 9–12). First, the clinical symptoms vary so much that it often mimics or is mistaken for other conditions (1, 2). Because no single diagnostic test is at hand, SLE is currently diagnosed when at least 4 of 11 complex, clinical criteria, as defined by the American College of Rheumatology (13, 14), are fulfilled. Second, the course of the disease is characterized by alternating periods of flares and remissions (1, 2). There are no biomarkers at hand for predicting and/or identifying the start and end of a flare, which would be a key feature for optimizing treatment (1, 2, 5). Third, the therapeutic regime could be even further optimized if validated biomarkers for stratifying the patients into clinical phenotypic subsets, reflecting disease severity (15), were available. Fourth, the absence of markers has significantly hampered the efforts to monitor and evaluate the effects of (novel) therapeutics (6, 16). Considering the complexity of SLE, it is reasonable to argue that more than one biomarker signature will be required in order to reflect all aspects of SLE (6). Hence, the need to define molecular portraits associated with SLE is significant.

Compared with SLE (inflammatory phenotype) (1, 2), SSc displays a less anti-inflammatory and more fibrotic phenotype (4, 7, 17). This disorder, which has a prevalence of about 3 to 24 cases per million persons (18), is as SLE, diagnosed by evaluating an intricate pattern of clinical features. Based on the pattern of skin involvement (19), SSc is commonly classified into two subsets, limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc). As for SLE, the need for specific biomarkers of SSc for diagnosis, classification, prog-

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[1] The abbreviations used are: SLE, systemic lupus erythematosus; ANA, anti-nuclear antibodies; AUC, area under the curve; CRP, C-reactive protein; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; ROC, receiver operating characteristics; scFv, single-chain fragment variable; SLEDAI-2K, SLE disease activity index; SSc, systemic sclerosis; SVM, support vector machine.

nosis, and for monitoring the response to therapy is significant (8, 20).

Considering the nature of SLE (1, 2) and SSc (3, 8, 17), deciphering the serum, plasma, and/or urine proteomes, would shed further light on these diseases, and could provide the candidate biomarker signatures much longed for (6, 8, 10–12, 20). Despite major efforts, using a plethora of methods, including conventional proteomic technologies, such as two-dimensional gels and mass spectrometry, our knowledge about the serum, plasma, and urine signatures reflecting SLE (6, 10–12) and SSc (3, 7, 8, 20) is still very limited, and mainly restricted to single laboratory variables displaying inadequate specificity and sensitivity. Targeting crude proteomes, such as serum, has proven challenging using standard proteomic approaches because of sample complexity and methodological shortcomings (21–23).

In recent years, affinity proteomics, mainly represented by antibody-based microarrays, have been established as a technology capable of performing multiplex profiling of complex proteomes in a sensitive manner (24–26). In this context, we have developed a state-of-the-art recombinant antibody microarray technology platform (24, 27, 28) and validated its use within disease proteomics (24, 29–32). Focusing on various cancers (30–33) and inflammatory conditions (31) (Wingren *et al.*, unpublished observations), we have deciphered candidate serum/plasma and tissue biomarker signatures for *e.g.* disease diagnosis, prognosis, and classification, as well as for monitoring the molecular effects of therapy and for selecting patients eligible for therapy.

In this proof-of-concept study, we have explored the potential of our recombinant antibody microarray set-up for profiling the serum proteome of SLE and SSc, targeting high- and low-abundant immunoregulatory proteins in crude, directly biotinylated sera. The data showed that several SLE-associated candidate serum protein signatures could for the first time be identified reflecting disease, disease severity (phenotypic subsets), and disease activity. Although SLE and SSc could be differentiated, the data implied that the serum profiles of SSc *versus* controls were more similar. Hence, this study demonstrated that a minimally invasive blood sample harbored disease-specific information reflecting autoimmune connective tissue diseases, further enhancing our fundamental understanding of SLE and SSc.

MATERIALS AND METHODS

Clinical Samples—This study was approved by the regional ethics review board in Lund, Sweden. In total, 65 serum samples were collected at Lund University Hospital (Lund, Sweden), including 20 SSc patients (Table I), 30 SLE patients (Table II), and 15 healthy volunteers (mean age 44 (range 20–69), gender female:male 14:1). The SLE and SSc patients were all recruited from the Department of Rheumatology (Lund University Hospital) (34, 35). Serum samples of the SSc patients were collected at the first enrolment for inquiry of the SSc diagnosis, and the demographics are described in Table I. The SSc patients fulfilled the inclusions criteria of (a) a definitive diagnosis of SSc according to the ACR (36); (b) a disease duration of less than 5 years from the onset of skin involvement; and (c) had not been previously treated with any of the following drugs: azathioprine, chlorambucil, colchicine, cyclophosphamide, cyclosporine S, D-penicillamine, methotrexate, or mycophenolate mofetil. The SSc patients were grouped according to whether they had limited cutaneous SSc (lcSSc, $n = 10$) or diffuse cutaneous SSc (dcSSc, $n = 10$) (19), and the clinical data were obtained as previously described (37). The SLE patients had clinical SLE diagnosis and displayed four or more ACR classification criteria (13, 14). The serum samples were collected during follow-up when the patients were presented with a flare. The demographics are described in Table II. The SLE patients were grouped according to disease severity as previously described (15): (1) skin and musculoskeletal involvement (SLE1, $n = 10$); (2) serositis, systemic vasculitis but not kidney involvement (SLE2, $n = 10$); and (3) presence of SLE glomerulonephritis (SLE3). The clinical disease activity was defined as SLEDAI-2K score (38). All samples were aliquoted and stored at −80 °C until further use. A second, independent patient cohort, containing 20 SLE3 patients and 20 healthy volunteers, collected at Lund University Hospital (Lund, Sweden) (the demographics are described in supplemental Table 1) were used to validate selected differentially expressed analytes, including ≤10 cytokines (low-abundant markers) and one complement protein (high-abundant marker).

TABLE I
Demographic data of the SSc patients included in the study

Parameter	lcSSc	dcSSc
No.	10	10
Gender (female:male)	10:0	7:3
Age at onset	51 (44–70)[a]	47 (34–55)[a]
Duration (months)	31 (24–43)[a]	17 (10–28)[a]
Skin score	10 (9–12)[a]	17 (10–28)[a]
Organ involvement		
Esophagus	10	9
Lung	5	7
Heart	1	4
PAH	1	0
Kidney	1	1
Muscle	0	1
Joint	1	1
No. ANA positive	10	10
No. anti-Scl-70 positive	0	4
No. anti-centromere positive	8	0
No. ESR raised	2	5
No. CRP raised	3	6
No. IgG raised	2	4

[a] Median (25th and 75th percentile).

Labeling of Serum Samples—The serum samples were labeled using previously optimized labeling protocols for serum proteomes (27, 28, 39). Briefly, the serum samples were diluted 1:45 in phosphate-buffered saline (PBS), resulting in a concentration of about 2 mg/ml, and biotinylated at a molar ratio of biotin:protein of 15:1 using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL). Unreacted biotin was removed by dialysis against PBS for 72 h, using a 3.5 kDa molecular weight (MW) dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA). The samples were aliquoted and stored at −20 °C.

Production and Purification of Single-chain Fragment Variable (scFv)—One hundred thirty-five human recombinant single-chain fragment variable (scFv) antibody fragments directed against 60 different analytes mainly involved in immunoregulation, anticipated to reflect the events taking place in SLE and SSc (supplemental Table 1), were selected from the n-CoDeR library (40) and kindly provided by

TABLE II
Demographic data of the SLE patients included in the study

Parameter	SLE1	SLE2	SLE3
No.	10	10	10
Gender (female:male)	10:0	8:2	8:2
Age at onset[a]	45 (26–51)	38 (27–46)	28 (24–34)
Duration (months)[a]	102 (42–179)	79 (15–206)	74 (10–139)
SLEDAI-2K[b]	6 (4–10)	7 (2–32)	16 (10–32)
ANA (titre)[b]	217 (0–3200)	217 (14–1600)	217 (0–1600)
Anti-DNA (% positive)[c]	10	60	70
Complement protein C1q (%)[b]	105.5 (58–217)	109 (0–133)	58.5 (35–158)
Complement protein C3(mg/ml)[b]	1.10 (0.35–1.48)	0.85 (0.37–1.56)	0.59 (0.34–1.22)
Complement protein C4 (mg/ml)[b]	0.16 (0.09–0.43)	0.13 (0–0.28)	0.11 (0.04–0.33)
CRP[b]	12 (0–89)	13.5 (0–75)	11.5 (0–33)
Treatment			
Prednisolon	4	8	0
Hydrochloroquine	5	4	2
Azathoprine	2	4	1
Cyclosporine S	1	0	3
Cyclosphosphamide	1	0	3
Methotrexate	0	0	1
Intravenous Ig	0	0	1
Definition			
SLE1	Skin and musculoskeletal involvement		
SLE2	Serositis, systemic vasculitis, but not kidney involvement		
SLE3	SLE glomerulonephritis		

[a] Median (25th and 75th percentile).
[b] Median ± range.
[c] Mean.

BioInvent International AB (Lund, Sweden). The specificity, affinity (normally in the nM range), and on-chip functionality of these phage display derived scFv antibodies (40) was ensured by using (i) stringent phage-display selection protocols (40), (ii) multiple clones (≤4) per target analyte, and (iii) a molecular design, adapted for microarray applications (24, 41) (Wingren *et al.*, unpublished observations). The specificity of several of the antibodies have previously also been validated using well-characterized, standardized serum samples, and orthogonal methods, such as mass spectrometry, ELISA, MSD, CBA, and MS, as well as using spiking and blocking experiments (supplemental Table 2). All scFv antibodies were produced in 100 ml *E. coli* cultures and purified from expression supernatants, using affinity chromatography on Ni-NTA agarose (Qiagen, Hilden, Germany). Bound molecules were eluted with 250 mM imidazole, extensively dialyzed against PBS, and stored at 4 °C, until further use. The protein concentration was determined by measuring the absorbance at 280 nm (average concentration 480 μg/ml, range 100 to 800 μg/ml). The degree of purity and integrity of the scFv antibodies were evaluated by 10% SDS-PAGE (Invitrogen, Carlsbad, CA).

Fabrication and Processing of Antibody Microarrays—For production of the antibody microarrays, we used a set-up previously optimized and validated (27, 28, 30, 32). Briefly, the scFv microarrays were fabricated, using a noncontact printer (Biochip Arrayer, Perkin Elmer Life and Analytical Sciences). The antibodies were spotted onto black polymer MaxiSorb microarray slides (NUNC A/S, Roskilde, Denmark), resulting in an average of 11 fmol scFv per spot (range 2–19 fmol). Eight replicates of each scFv clone were arrayed to ensure adequate statistics. The on-chip performance of the antibodies has been previously validated (27, 30–32, 41), for review see (41–44). In total, 160 antibodies and controls were printed per slide orientated in two columns with 8 × 80 spots per column.

For handling of the arrays, we used a protocol recently optimized (33). Briefly, the slides were manually blocked in 5% (w/v) fat-free milk powder (Semper AB, Sundbyberg, Sweden) in PBS, and then placed in a Protein Array Work station (Perkin Ellmer Life and Analytical Sciences) for automated handling. The slides were washed with 0.5% (v/v) Tween-20 in PBS. The biotinylated serum sample was diluted 1:2 (resulting in a total serum dilution of 1:90) in 1% (w/v) fat-free milk powder and 1% (v/v) Tween in PBS (PBS-MT) prior to incubation on the array. The arrays were visualized with 1 μg/ml Alexa-647 conjugated streptavidin diluted in PBS-MT. Finally, the arrays were dried under a stream of nitrogen gas and scanned with a confocal microarray scanner (ScanArray Express, Perkin Elmer Life and Analytical Sciences) at 5-μm resolution, using three different scanner settings. The ScanArray Express software V3.0 (Perkin Elmer Life and Analytical Sciences) was used to quantify the intensity of each spot, using the fixed circle method. The local background was subtracted to compensate for possible local defects, the two highest and the two lowest replicates were automatically excluded and each data point represents the mean value of the remaining four replicates. The coefficient of correlation for intra-assays was >0.99 and for interassays >0.96, respectively.

Data Normalization—Only nonsaturated spots were used for further analysis of the data. Chip-to-chip normalization of the data sets was performed, using a semiglobal normalization approach (24, 30, 32), conceptually similar to the normalization developed for DNA microarrays. Thus, the coefficient of variation was first calculated for each analyte and ranked. Fifteen percent of the analytes that displayed the lowest coefficient of variation values over all samples were identified, corresponding to 21 analytes, and used to calculate a chip-to-chip normalization factor. The normalization factor N_i was calculated by the formula $N_i = S_i/\mu$, where S_i is the sum of the signal intensities for the 21 analytes for each sample and μ is the sum of the signal intensities for the 21 analytes averaged over all samples. Each data set generated from one sample was divided with the normalization factor N_i. For the intensities, log2 values were used in the analysis.

Data Analysis—The support vector machine (SVM) is a supervised learning method in R (45–47) that we used to classify the samples. The supervised classification was performed using a linear kernel, and the cost of constraints was set to 1, which is the default value in the R function SVM, and no attempt was performed to tune it. This absence of parameter tuning was chosen to avoid overfitting. The SVM was trained using a leave-one-out cross-validation procedure. Briefly, the training sets were generated in an iterative process in which the samples were excluded one by one. The SVM was then asked to blindly classify the left out samples as either disease or healthy, and to assign a SVM decision value, which is the signed distance to the hyperplane. No filtration on the data was done before training the SVM, *i.e.* all antibodies used on the microarray were included in the analysis. Further, a receiver operating characteristics (ROC) curve, as constructed using the SVM decision values and the area under the curve (AUC), was calculated. AUC values were in general interpreted as 0.5–0.6 = poor; 0.6–0.7 = fair; 0.7–0.8 = medium; 0.8–0.9 = good; 0.9–1.0 = excellent. The discriminatory power of clinical laboratory parameters (anti-DNA antibodies, antinuclear antibodies (ANA), complement protein C1q, complement protein C3, complement protein C4, C-reactive protein (CRP), and/or SLEDAI-2K) were evaluated single predictors or in combination by using the same leave-one-out SVM procedure as for the array data.

Significantly up- or down-regulated plasma proteins ($p < 0.05$) were defined based on the relative protein levels and identified using Wilcoxon signed-rank test, log transformed and mean centered. It should be noted that the approach did not differentiate whether the observed up- or down-regulated levels of an analyte was because of an increased or decreased production or an increased or decreased consumption. The samples were visualized using Qlucore (Lund, Sweden) or R (heat maps).

Finally, we investigated the impact of the small sample groups at the end results of the statistical analysis. To this end, we randomly changed the group labels across the samples, and re-ran the classification using the randomized (permutated) groups. The randomization process was repeated 20 times for each classification. The analysis showed that the even though the sample groups were small, the classifier performed among the best when the groups were made up of their original, correct members (when the original group displayed an adequate ROC AUC value) (supplemental Fig. 1A). Thus, the data showed that the successful classification adequately reflected the original stratification, and was not the results of a confounding factor derived from the small sample groups. In the cases where SVM models also were built using data subsets composed of only significant analytes, a corresponding analysis was performed (supplemental Fig. 1B). Here, each permutated model was constructed using only the analytes found to be significant for each individual permutation, *i.e.* the analytes selected for training and testing each permutation was identified using the same criterion (wilcoxon p value < 0.05) as for the original stratification.

Validation of Array Data—A human Th1/Th2 10-plex MSD (Meso Scale Discovery, Gaithersburg, MD) assay was run in an attempt to validate the antibody microarray results (differentially expressed analytes), focusing on SLE. Both patient cohorts of SLE were profiled using MSD (supplemental Figs. 2–6). Each well of the MSD 96-plate had been prefunctionalized with antibodies against IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, and TNF-α in spatially distinct electrode spots. The assay was run according to the protocol provided by the manufacturer and the electrochemiluminiscence-based readout was performed in an MSD SECTOR® instrument. The limit of detection was defined as 2.5 times the standard deviation of the zero point in the standard curve. The MSD assay was applied to both patient cohorts.

Further, the levels of three complement proteins, including C1q, C3, and C4, were determined by an electroimmuno-, turbidometric-, and/or nephelometric-based assay in the first patient cohort (supplemental Figs. 2 and 6). Finally, the levels of complement protein C3 was determined in both patient cohorts using our array set-up (supplemental Figs. 3 and 5).

RESULTS

Profiling of SLE and SSc Serum Proteomes—First, we determined a focused serum proteome profiles of SLE and SSc, and compared them to that of the healthy controls. The top ≤25 significantly ($p < 0.05$) expressed nonredundant analytes are shown as heat maps in Fig. 1. Although only four analytes (*e.g.* IFN-γ, and IL-4) were found to be differentially expressed in SSc (Fig. 1*A*), the data showed that the expression patterns of 40 analytes differed between SLE *versus* controls, including both down-regulated (*e.g.* C1q, C3, and C5) and up-regulated analytes (*e.g.* IL-6, IL-10, IFN-γ, and TNF-α) (Fig. 1*B*). It should be noted that we could not differentiate whether the observed up- and down-regulated levels of an analyte was because of an increased or decreased production or increased or decreased consumption.

In order to evaluate the ability of the set-up to differentiate SSc and SLE *versus* controls based on the observed protein expression profiles, we ran a leave-one-out cross-validation based on all antibodies with a SVM, and collected the decision values for all samples. These prediction values were then used to construct a ROC curve, and the AUC value was calculated (Fig. 1). The results showed that SLE *versus* controls could be reasonably differentiated (AUC = 0.76) (Fig. 1*B*), with a specificity and sensitivity of 0.80% and 0.67%, respectively. Using orthogonal assays, targeting low-abundant markers (≤10 cytokines; MSD-based assay) (supplemental Fig. 2A) and high-abundant markers (≤3 complement proteins; electroimmuno-, turbidometric-, and/or nephelometric-based assays) (supplemental Fig. 2B), differentially expressed analytes were successfully validated. In contrast, SSc *versus* controls could not be distinguished (AUC = 0.47) (Fig. 1*A*).

Next, we examined whether a multiplexed serum profile differentiating SLE *versus* SSc could be determined (Fig. 1*C*). The data showed that 42 analytes were significantly differentially expressed of which all, but 3 (C1q, C3, and mucin-1), were up-regulated in SLE (*e.g.* IL-6, IL-12, IFN-γ, and TNF-α). Although the discriminatory power was poor (AUC = 0.59) using unfiltered data, *i.e.* all antibodies, the AUC value was found to be 0.69 when only the differentially expressed analytes were used.

Serum Protein Profiles of SSc Phenotypic Subsets—In an attempt to further refine the serological portrait of SSc, we determined the expression profiles of the two commonly recognized phenotypic subsets, dcSSc and lcSSc (Fig. 2). The data showed that seven significantly differentially expressed analytes were observed for dcSSc *versus* controls (the most prominent being IFN-γ and IL-4), five for lcSSc *versus* controls

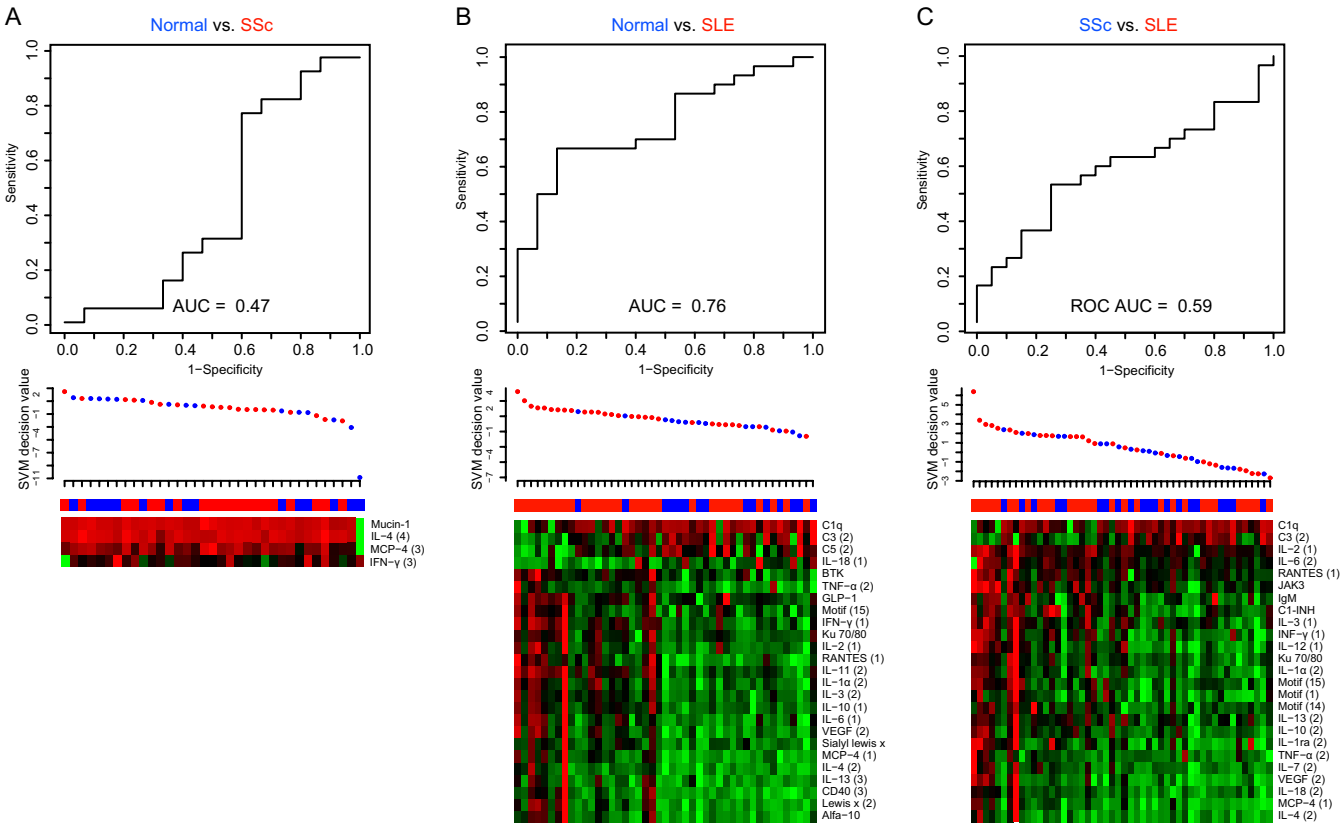

FIG. 1. **Expression profiling of serum proteomes from SSc and SLE using a 135-recombinant-antibody microarray.** The samples were classified using a leave-one-out cross validation approach with a SVM, and illustrated as ROC curves. The patients are ordered by decreasing decision value as assigned by the SVM classifier (middle panel). Differentially expressed analytes are shown in heat maps: green, down-regulated; red, up-regulated; and black, equal levels. *A*, Classification of healthy controls (blue) *versus* SSc (red). Four differentially expressed analytes, recognized by five antibodies, were identified. *B*, Classification of healthy controls (blue) *versus* SLE (red). Forty nonredundant differentially expressed analytes, recognized by 58 antibodies were identified, of which the 25 highest ranked, *i.e.* significantly differentially expressed (p values), analytes are shown. *C*, Classification of SSc (blue) *versus* SLE (red). Forty-two nonredundant differentially expressed analytes, recognized by 62 antibodies were identified, of which the 25 highest ranked analytes (p values) are shown.

(*e.g.* IL-4, MCP-4, and CD-40), whereas none could be observed for dcSSc *versus* lcSSc. Using unfiltered data, the phenotypic subsets could neither be differentiated from the controls nor from each other (AUC = 0.50). In contrast, based on the differentially expressed analytes only, both dcSSc (AUC = 0.71) and lcSSc (AUC = 0.90) could be distinguished from the controls.

Classification of SLE—We then determined the serum protein profile of three phenotypic subsets of SLE grouped according to increased disease severity, SLE1 < SLE2 < SLE3, and compared them with that of the controls (Fig. 3). The results showed that the number of differentially expressed analytes (15 < 28 < 44) and AUC values (0.55 < 0.67 < 0.99) increased with the severity of the symptoms.

In more detail, in the case of SLE1, complement proteins were found to be down-regulated (*i.e.* found at lower levels) (*e.g.* C1q, C3, C5, and Factor B), whereas several cytokines were found to be up-regulated (*e.g.* IL-4, IL-6, IL-10, TNF-α) (Fig. 3*A* and 3*D*). Despite the observed differences in serum protein expression patterns, SLE1 *versus* controls could not be discriminated (AUC = 0.55). Although complement proteins were down-regulated in SLE2 as well (*e.g.* C1q), the pattern of up-regulated cytokines contained a mixture of both Th1 cytokines (*e.g.* IL-2, IL-12, IFN-γ, and TNF-α) and Th2 cytokines (*e.g.* IL-6, IL-10) (Figs. 3*B* and 3*D*). The discriminatory power of this serum biomarker profile was moderate (AUC = 0.67), corresponding to a specificity of 60% and sensitivity of 80%. In contrast, SLE3 *versus* controls could readily be discriminated ($p = 6\times10^{-7}$; AUC = 0.99) with a sensitivity and specificity of 100 and 93%, respectively (Figs. 3*C*). Of note, in the training of the SVM, no filtration of data was made, *i.e.* all the measurements were included. Apart from the complement proteins (*e.g.* C1q, C3, and C5) and IL-18 (down-regulated), the pattern of 44 differentially expressed analytes contained mainly up-regulated cytokines, including both Th1 and Th2 cytokines (Figs. 3*C* and 3*D*). For all three comparisons, a set of differentially expressed markers (cytokines) were successfully validated using a 10-plexed MSD-based assay (supplemental Figs. 3*A*–3*C*). Furthermore, the differential expression pattern observed for both a high-

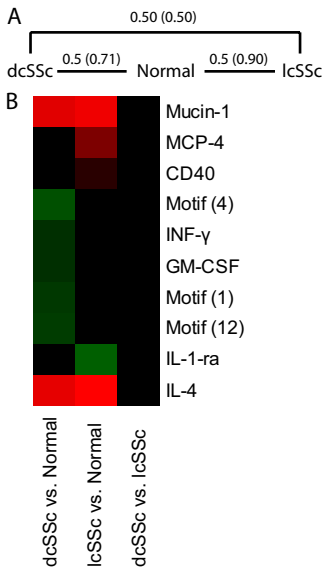

FIG. 2. **Expression profiling of serum proteomes from clinical subsets of SSc, dcSSc, and lcSSc, using a 135-recombinant-antibody microarray.** *A*, Classification of dcSSc and lcSSc *versus* healthy controls based on all 135 antibodies, using a SVM-based leave-one-out cross validation test, expressed in terms of AUC values. AUC values obtained when using only significantly differentially expressed analytes are given within brackets. *B*, Significantly differentially expressed analytes are shown in a heat map. Seven differentially expressed analytes, recognized by seven antibodies were identified for dcSSc *versus* controls; five analytes, recognized by five antibodies for lcSSc *versus* controls; whereas none were observed for dcSSc *versus* lcSSc. Green, down-regulated; red, up-regulated; and black, equal levels. The color represent the fold change of a particular marker across all samples within each sample cohort, calculated using the average signal intensities.

abundant marker (complement protein C3) and a set of low-abundant markers (cytokines) were also validated on an independent, second patient cohort (SLE3 *versus* healthy controls) using both array technology and a MSD-based assay (supplemental Figs. 3*D* and 3*E*). Hence, the data showed that first candidate SLE serological biomarker signatures reflecting disease severity had been delineated using recombinant antibody microarrays.

Refined Classification of SLE versus SSc—Considering the fact that the phenotypes of SLE were reflected in the serum profiles, we compared the molecular pattern of the phenotypic subsets of SLE with that of SSc to refine the observed serological differences between these conditions (Fig. 4). As the subsets of SSc could not be differentiated (Fig. 2), SSc was kept as one cohort. The data showed that the number of significantly differentially expressed analytes ($3 < 36 < 49$) and AUC values ($0.40 < 0.69 < 0.98$) for SLE phenotypic subsets *versus* SSc increased with the severity of SLE (Fig. 4).

In the case of SLE *versus* SSc, only three differentially expressed analytes (C1q, C3 and TNF-α) were detected, and the cohorts could not be differentiated (AUC = 0.40) (Figs. 4*A* and 4*D*). In contrast, SLE2 *versus* SSc could be differentiated (AUC = 0.69) with a 75% specificity and 60% sensitivity, and 36 differentially expressed analytes were observed (Figs. 4*B* and 4*D*). Except for mucin-1 and Ku 70/80 (down-regulated), a pattern of mainly up-regulated analytes was observed for SLE2, including both Th1 and Th2 cytokines. Further, SLE3 *versus* SSc was readily classified (AUC = 0.98; $p = 6\times10^{-7}$), displaying a sensitivity and specificity of 100 and 85%, respectively (Fig. 4*C*). Although complement proteins (*e.g.* C1q, C3, and C5) and TNF-β were found to be down-regulated (*i.e.* found at lower levels), a majority of the 49 significantly differentially expressed analytes were found to be up-regulated in SLE3 and composed of a panel of both Th1 and Th2 cytokines (Figs. 4*C* and 4*D*). Hence, the serological portraits differentiating SLE and SSc had been further deciphered and refined (cfs. Figs. 1 and 4).

Refined Classification of SLE Reflecting Disease Severity—Next, we further investigated whether the SLE samples could be classified according to disease severity, determined á priori based on an intricate pattern of clinical parameters (Fig. 5). To this end, we compared the serum protein profiles of the three SLE phenotypic subsets with each other. The results showed that SLE1 (least symptoms) *versus* SLE3 (most symptoms) could be distinguished (AUC = 0.80), corresponding to a specificity and sensitivity of 70% and 80%, respectively (Fig. 5*A*). Moreover, SLE1 *versus* SLE2 could also be separated (AUC = 0.73), whereas SLE2 *versus* SLE3 (AUC = 0.58) appeared to be more similar. Notably, these comparisons were all based on unfiltered data. When only the differentially expressed analytes were used, SLE1 *versus* SLE2 and SLE2 *versus* SLE3 could also be classified, displaying AUC values of 0.79 and 0.81, respectively. The relationship between the three clinical phenotypes is further highlighted in Fig. 5*B*, where a principle component analysis, based on the 13 most significantly differentially expressed analytes is shown. Again, the data outlined that the three cohorts could be classified according to disease severity (phenotype), indicating that serological profiles had been delineated that could be used for phenotype classification. In addition, the array data was also found to display a better classification than single or combined conventional clinical parameters, including, ANA, anti-DNA, SLEDAI-2k, C1q, C3, C4, and CRP, in a majority of the cases (cfs. Fig. 5 and Table III).

In more detail, 23 significantly differentially expressed analytes were observed for SLE1 *versus* SLE2 (Fig. 5*C*). Apart from five up-regulated analytes (*e.g.* IL-4 and TNF-β), the markers were down-regulated (*e.g.* IL-10 and IL-12) in SLE1. Although only seven differentially expressed (down-regulated) analytes (*e.g.* IFN-γ, IL-6, IL-10, and IL-13) were detected for SLE2 *versus* SLE3, a total of 32 analytes were detected for SLE1 *versus* SLE3. In the latter case, all but two analytes (IL-3 and IL-1β), were found to be down-regulated in SLE1. Notably, the Th1 cytokines IL-12, IFN-γ, and IL-2 increased as the disease progressed from SLE1 through SLE2 to SLE3, whereas central Th2 cytokines either decreased (IL-4) or

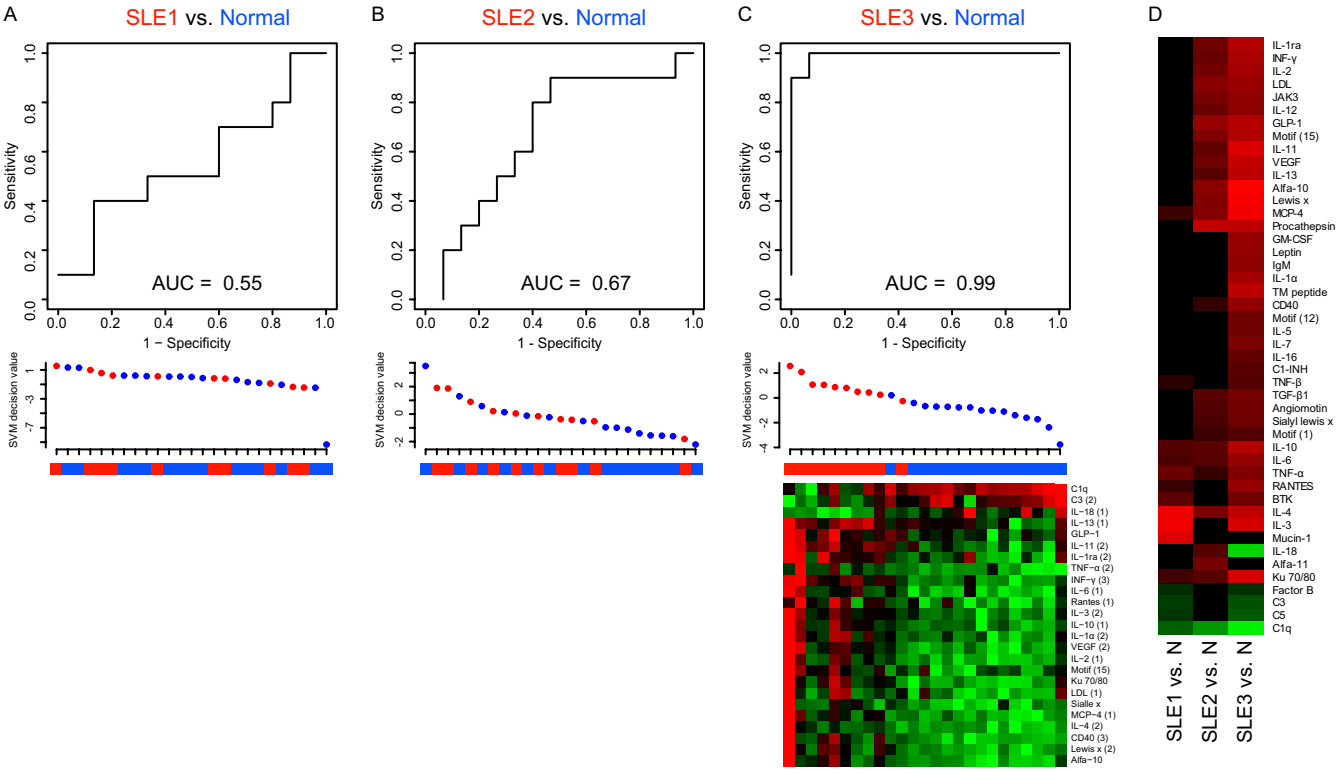

FIG. 3. **Expression profiling of phenotypic subsets of SLE reflecting increased disease severity, including SLE1 (least symptoms), SLE2, and SLE3 (most severe symptoms), using a 135-recombinant-antibody microarray.** The samples were classified using a leave-one-out cross validation approach with a SVM, and illustrated as ROC curves. The patients are ordered by decreasing decision value as assigned by the SVM classifier (*middle panel*). Differentially expressed analytes are shown in heat maps: green, down-regulated; red, up-regulated; and black, equal levels. The color represent the fold change of a particular marker across all samples within each sample cohort, calculated using the average signal intensities. *A*, Classification of SLE1 (red) *versus* controls (blue). *B*, Classification of SLE2 (red) *versus* controls (blue). *C*, Classification of SLE3 (red) *versus* controls (blue). The top 25 highest ranked, *i.e.* significantly differentially expressed (*p* values), analytes are shown. *D*, Fifteen significantly differentially expressed analytes, recognized by 17 antibodies, were identified for SLE1 *versus* controls; 29 analytes, recognized by 36 antibodies for SLE2 *versus* controls; and 44 analytes recognized by 72 antibodies for SLE3 *versus* controls.

showed moderate increase (IL-5 and IL-10) (Fig. 5*D*), indicating a potentially Th1 skewed immune response as the severity of SLE progressed. Finally, a set differentially expressed markers (cytokines) were successfully validated for all three comparisons using a 10-plexed MSD-based assay (supplemental Fig. 4), further highlighting the findings.

Patients Can be Classified According to Disease Activity—Finally, we investigated whether serological biomarker signatures reflecting SLE disease activity could de-convoluted. To this end, the SLE samples were grouped into three groups based solely on their disease activity, as reflected by their SLEDAI-2K values; low (3–6), mid (9–19) and high (22–34).

The antibody microarray data showed that low *versus* mid SLE activity samples could not be distinguish using unfiltered data (AUC = 0.53), whereas the AUC value was found to reach 0.82 when only the 12 differentially expressed analytes were used (Fig. 6*A*). Except for IL-18, only down-regulated analytes (*e.g.* IL-8, IL-10, IL-12, and IFN-γ) were observed in low *versus* mid SLE activity (Fig. 6*B*). A set of differentially expressed markers (cytokines) were validated using an MSD-based assay (supplemental Fig. 5A). In contrast, mid *versus* high SLE activity could be differentiated with very high confidence based on nonfiltered data, as reflected by an AUC of 0.93, corresponding to a specificity and sensitivity of 91% and 100%, respectively (Fig. 6*A*). Among the eight differentially expressed analytes, five were up-regulated (*e.g.* C1q, C4, IL-8, and IL-12) and three down-regulated (IL-1-ra, IL-18, and TNF-α) in mid *versus* high SLE activity (Fig. 6*B*). A set of differentially expressed analytes (cytokines) were successfully validated using a MSD-based assay (supplemental Fig. 5B). Moreover, low *versus* high SLE activity could be moderately differentiated (AUC = 0.70) with a specificity of 82% and sensitivity of 67%, showing a panel of 10 differentially expressed analytes, including five up-regulated complement proteins (C1q, C3, C4, C5 and Factor B) and five down-regulated analytes (*e.g.* CD40, IL-6, and IFN-γ) (Figs. 6*A* and 6*B*). A set of differentially expressed markers (cytokines) were again validated using a MSD assay (supplemental Fig. 5C). Focusing on the complement proteins, the trends showed that the levels of complement proteins decreased as the

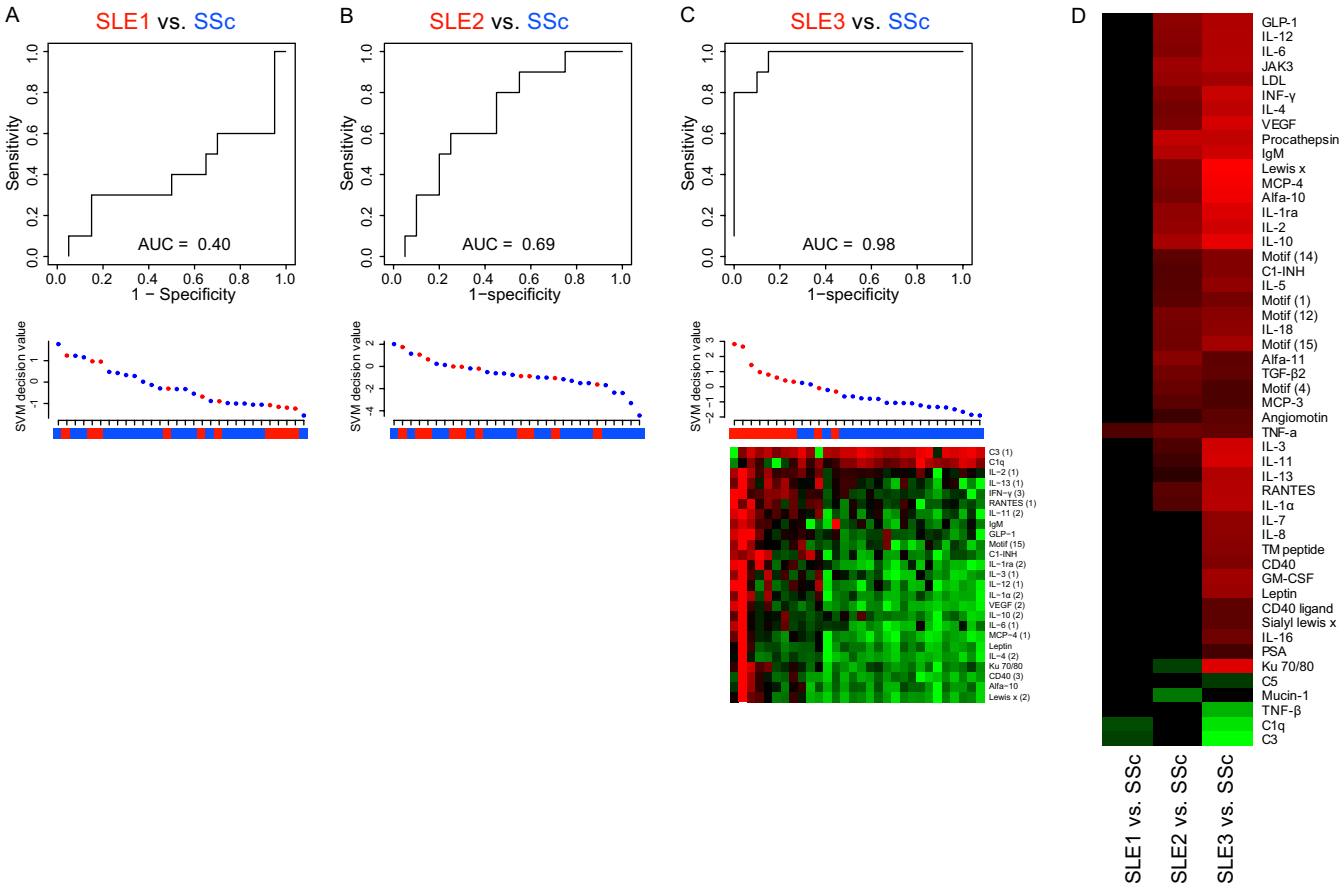

FIG. 4. **Expression profiling of phenotypic subsets of SLE reflecting increased disease severity and SSc, using a 135-recombinant-antibody microarray.** The samples were classified using a leave-one-out cross validation approach with a SVM, and illustrated as ROC curves. The patients are ordered by decreasing decision value as assigned by the SVM classifier (*middle panel*). Differentially expressed analytes are shown in heat maps: green, down-regulated; red, up-regulated; and black, equal levels. The color represent the fold change of a particular marker across all samples within each sample cohort, calculated using the average signal intensities. *A*, Classification of SLE1 (red) *versus* SSc (blue). *B*, Classification of SLE2 (red) *versus* SSc (blue). *C*, Classification of SLE3 (red) *versus* SSc (blue). The top 25 highest ranked, *i.e.* significantly differentially expressed (*p* values), analytes are shown. *D*, Three significantly differentially expressed analytes, recognized by three antibodies, were identified for SLE1 *versus* SSc; 36 analytes, recognized by 47 antibodies for SLE2 *versus* SSc; and 49 analytes recognized by 79 antibodies for SLE3 *versus* SSc.

activity of the disease increased, as reflected by C1q, C3, C4, and C5 (Figs. 6*C* and 6*D*). In addition, the pattern of differentially expressed C1q, C3, and C4 was successfully validated using electroimmuno-, turbidometric-, and/or nephelometric-based assays. Hence, the data indicated that the first generation of multiplexed serum profiles reflecting SLE disease activity could be identified. Of note, the array data was also found to display a better classification than single or combined conventional clinical parameters, including, ANA, anti-DNA, SLEDAI-2k, C1q, C3, C4, and CRP, in a majority of the cases (cfs. Fig. 6 and Table III).

In order to eliminate any influence of the disease severity on the observed serum profiles (Fig. 5), we re-ran the analysis above focusing on the SLE3 samples only (Fig. 7). The SLE3 samples were then divided into two groups, based on their SLEDAI-2K values; low (mean 13, range 10–16) and high (mean 24, range 17–32). The data showed that the SLE3 samples could be correctly classified according to disease activity, as reflected by an AUC value of 1.00, corresponding to a 100% specificity and 100% sensitivity (Fig. 7). The 20 significantly differentially expressed analytes are shown as a heatmap. Except for IL-4 and IL-1β, a pattern of mainly up-regulated analytes (*e.g.* CD40, IL-2, and IL-10) was observed as the disease activity increased. Notably, a set of differentially expressed analytes (cytokines) were successfully validated using a 10-plexed MSD-based assay (supplemental Fig. 6A). Furthermore, the expression pattern of selected differentially expressed markers (cytokines) was also validated on an independent, second patient cohort using an MSD-based assay (supplemental Fig. 6B). Noteworthy, the array data was also found to display a better classification than single or combined conventional clinical parameters in all cases (cfs. Fig. 7 and Table III).

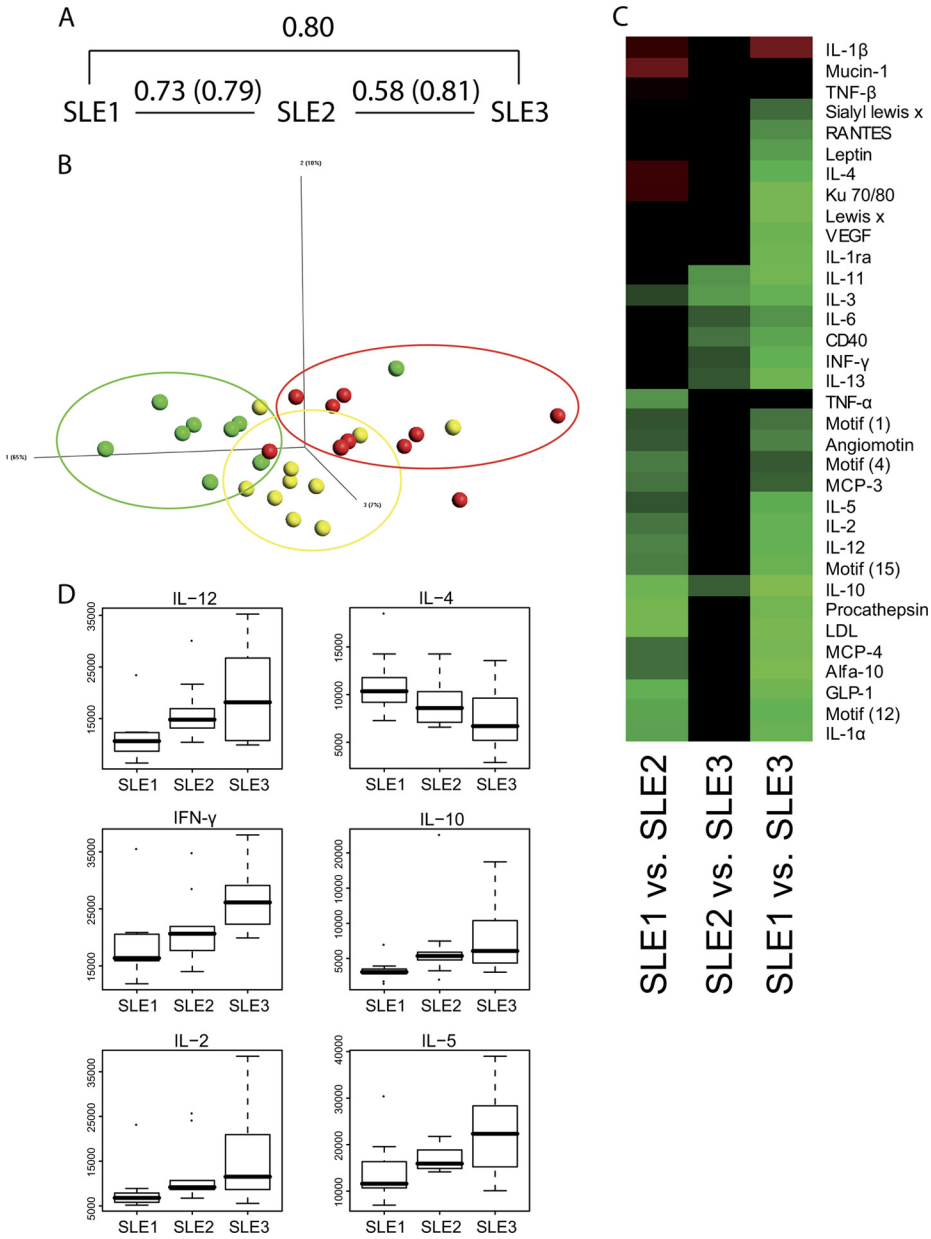

FIG. 5. **Expression profiling of phenotypic subsets of SLE reflecting increased disease severity, using a 135-recombinant-antibody microarray.** *A*, Classification of SLE1, SLE2 and SLE3 based on all 135 antibodies, using a SVM-based leave-one-out cross validation test, expressed in terms of AUC values. AUC values obtained when using only significantly differentially expressed analytes are given within brackets. *B*, PCA analysis of SLE1 (green), SLE2 (yellow), and SLE3 (red) based on the top 13 significantly differentially antibodies using Qlucore. *C*, Significantly differentially expressed analytes are shown in a heat map. Twenty-three differentially expressed analytes, recognized by 28 antibodies, were identified for SLE1 *versus* SLE2; seven analytes, recognized by eight antibodies for SLE2 *versus* SLE3; and 32 analytes recognized by 47 antibodies for SLE1 *versus* SLE3. Green, down-regulated; red, up-regulated; and black, equal levels. The color represent the fold change of a particular marker across all samples within each sample cohort, calculated using the average signal intensities. *D*, Microarray signal intensities observed for key Th1 (IL-2, IL-12, and IFN-γ) and Th2 (IL-4, and IL-10) cytokines shown as boxplots. The median values are indicated (thick line) and the hinges represent the 25th percentile and the 75th percentile, respectively.

In conclusion, we have shown that protein expression profiling of serum samples could be performed using recombinant antibody microarrays, enabling us to extract several unique biomarker signatures reflecting SLE and SSc and clinical subsets thereof.

DISCUSSION

Based on the notion that immunoregulation is a particular phenomenon in SLE and SSc, we have in this pilot study taken on an affinity proteomics approach in order to harness the diagnostic, prognostic, and classification power of the immune system to address these conditions. To this end, we designed our antibody microarray to target predominantly some of the key regulatory serum molecules. With this limited range of specificities ($n = 60$), we showed that affinity proteomics could still be used to extract disease-associated serum portraits for SSc and in particular SLE reflecting disease, disease severity, disease activity, and phenotypic subsets. In the case of SLE, selected differentially expressed markers were validated using orthogonal assays and a second, independent patient cohort. Although the data needs to be further validated in follow-up studies, targeting larger, independent cohorts of patient samples before it could be transferred into clinical practice (29), the results clearly outlined the biomarker signatures.

SSc is a multisystem fibrotic disorder often described with a Th2 skewed, anti-inflammatory immune response, although the Th1/Th2 balance in SSc is still a matter of controversy (4,

TABLE III

The discriminatory power of clinical laboratory parameters for classification and prognosis of SLE evaluated as single predictors or in combination by using a leave-one-out cross-validation SVM procedure as for the antibody array data

Clinical Laboratory Parameter	ROC AUC value						
	SLE1 versus SLE2	SLE1 versus SLE3	SLE2 versus SLE3	SLE low[a] versus SLE mid	SLE low[a] versus SLE high	SLE mid[a] versus SLE high	SLE3 low[b] versus SLE3 high
Anti-DNA antibodies	0.61	0.50	0.50	0.50	0.50	0.54	0.50
Anti-nuclear antibodies	0.50	0.81	0.69	0.50	0.50	0.61	0.68
Complement protein C1q	0.59	0.69	0.58	0.50	0.74	0.70	0.50
Complement protein C3	0.62	0.65	0.55	0.50	0.65	0.50	0.50
Complement protein C4	0.59	0.81	0.75	0.50	0.74	0.70	0.50
C-reactive protein (CRP)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
SLEDAI-2K	0.50	0.83	0.68	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]
All combined[c]	0.50	0.52	0.50	0.50	0.65	0.50	0.50

[a] SLE patients groupd on SLEDAI-2K.
[b] SLE3 patients grouped based on SLEDAI-2K.
[c] excluding CRP.
[d] n.a. = not applicable, as the sample groups were á priori divided based on SLEDAI-2K.

48). Considering the fibrotic pathology of SSc, it would be natural to expect increased levels of IL-4 (Th2 cytokine) and decreased expression of IFN-γ (48). In accordance, we found IL-4 to be up-regulated and IFN-γ to be down-regulated in SSc *versus* controls. Although this feature was prominent also for the clinical subset dcSSc, only IL-4 was up-regulated in lcSSc. To some extent this might reflect the fact that dcSSc is considered to be dominated by rapidly progressive fibrosis of the skin, lungs, and other internal organs, whereas lcSSc is dominated by vascular manifestations, and skin and organ fibrosis is generally limited and slow to progress (4).

We found very few serological markers to be differentially expressed in SSc *versus* healthy controls, indicating on small differences in this particular data set. As far as we know, the association of mucin-1 and MCP-4 with SSc is a novel finding. In previous studies, serological biomarkers, exemplified by auto-antibodies, not targeted here, have been found to be an important marker of SSc (8). Other circulating serum biomarkers that have recently been indicated to be central and/or potentially differentially expressed in SSc include *e.g.* TGF-β, IL-1α, IL-4, IL-6, IL-10, and IL-13 (7, 8, 49–51). Apart from IL-4, we were unable to replicate these previous reports. The reason for these discrepancies could include significant biological heterogeneity of SSc (3, 20, 49), the size of the patient cohorts, and/or inherent difficulties in reproducing exploratory findings generated at different sites using different proteomic approaches (29, 52–54). This clearly implicated the importance of launching large, multicenter studies using parallel complementary technologies for deciphering complex, heterogeneous diseases (8, 29, 52–54).

Diagnosing SLE and SSc is challenging, which could result in patients suffering unexplained symptoms and untreated disease (1–3, 6, 8, 20). The situation is further complicated by patients suffering from overlap syndromes in which the autoimmune disease could start out as SLE and then transforms into SSc, and *vice versa* (55, 56). In this proof-of-concept study, distinct features and candidate serum signatures differentiating SLE and SSc were delineated. Notably, we showed that the magnitude of the observed differences coincided with the severity of SLE. Although only three analytes were found to be differentially expressed in SLE1 (least symptoms) *versus* SSc, the up-regulation of TNF-α and to some extent, the decreased levels of complement proteins in SLE1 agreed well with previous reports (4, 6, 8, 12, 15, 20). As the severity of SLE increased, several other analytes that were anticipated to be over expressed in SLE (6, 12) were also detected, *e.g.* IL-2, IL-12, and IFN-γ. However, some analytes anticipated to be more associated with SSc than SLE (3, 4, 6, 12, 20), were also found to be up-regulated in SLE *versus* SSc, *e.g.* IL-4, IL-5, IL-10, and TGF-β, indicating that the view of describing SLE *versus* SSc simply as an inflammatory phenotype *versus* a fibrotic phenotype needs to be refined. In general, our data indicated a much more vigorous and broader (systemic) immune response in SLE than in SSc.

Focusing on SLE, the absence of serological biomarkers for diagnosis is significant (6). Panel(s) of auto-antibodies, erythrocyte-bound complement protein product C4d, complement receptor 1, and platelet-bound-C4d have been proposed, but their potential remains to be validated (6). Notably, we found 40 differentially expressed, nonredundant, circulating serum biomarkers, reflecting the disease, again indicating the potential of our approach. This list of variables was composed of novel markers, as well as some markers that had previously been reported to be associated with SLE, such as IL-4, IL-6, IL-10, IL-12, IFN-γ, C3 and C4 (6, 12, 57, 58), further supporting our observations. It should, however, be noted that these known variables had not previously been reported in the context of a candidate signature for SLE diagnosis, but mainly as single or a few combined markers potentially reflecting disease activity (6, 12, 57, 58). Further studies will be required to validate our candidate signature(s) and to extract and interpret the disease biology reflected by these serological predictors.

Subsequently, these findings were extended, and we report here a candidate serum-based signature for classifying SLE

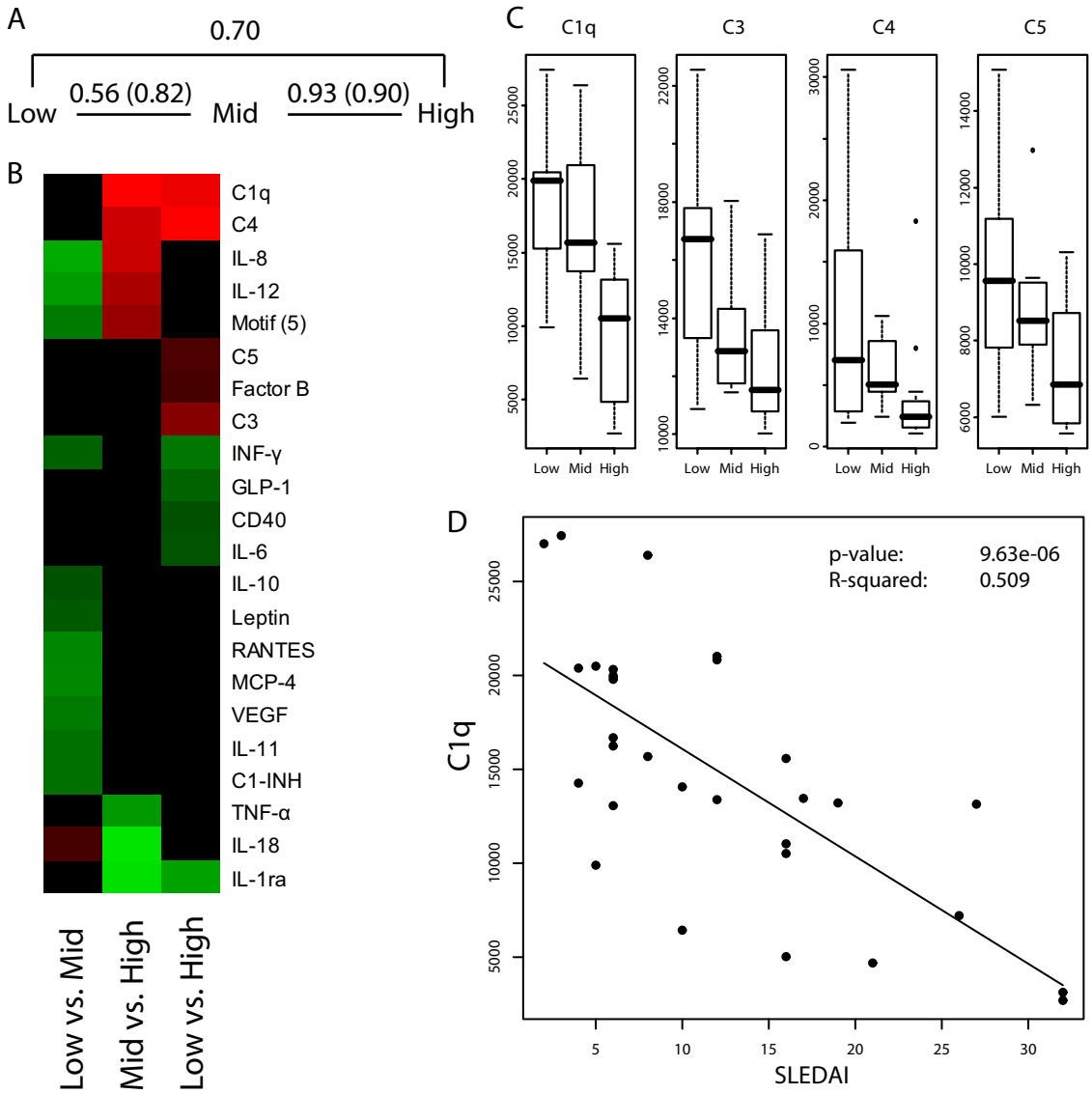

FIG. 6. **Expression profiling of subsets of SLE reflecting disease activity, using a 135 recombinant antibody microarray.** *A*, Classification of SLE, grouped based solely on disease activity (SLEDAI values); low (3 to 6), mid (9 to 19) and high (22 to 34), based on all 135 antibodies, using a SVM-based leave-one-out cross validation test, expressed in terms of AUC values. AUC values obtained when using only significantly differentially expressed analytes are given within brackets. *B*, Significantly differentially expressed analytes are shown in a heat map. Twelve differentially expressed analytes, recognized by 14 antibodies, were identified for low *versus* mid; eight analytes, recognized by 12 antibodies for mid *versus* high; and 10 analytes recognized by 16 antibodies for low *versus* high. Green, down-regulated; red, up-regulated; and black, equal levels. The color represent the fold change of a particular marker across all samples within each sample cohort, calculated using the average signal intensities. *C*, Microarray signal intensities observed for complement proteins, including C1q, C3, C4, and C5, shown as boxplots. The median values are indicated (thick line) and the hinges represent the 25th percentile and the 75th percentile, respectively. *D*, The correlation between array signal intensities for C1q and SLE disease activity, expressed as SLEDAI-2K.

according to disease severity (15). When we compared the serum portraits of the phenotypic subsets with those of the controls, the data showed, as could be expected, that the number of differentially expressed analytes and the discriminatory power of the candidate signature increased with the severity of SLE. As for example, our results implied that SLE3, lupus nephritis, *versus* controls could be differentiated with a sensitivity and specificity of 100% and 93%, respectively. Previous attempts to pin-point lupus nephritis has mainly relied on single or a few markers, although attempts have been made to identify multiplex signatures using *e.g.* mass spectrometry based approaches, but so far with limited success (6, 10, 11, 59). Further, our data implied a somewhat skewed Th2 response in SLE1, as IL-4, IL-6, and IL-10 were found to be up-regulated, although a mixture of Th1 and Th2 cytokines were up-regulated in SLE2 and SLE3, indicating a complex pattern of cytokine expression. This could, at least to some extent, reflect the complex and intricate disease pro-

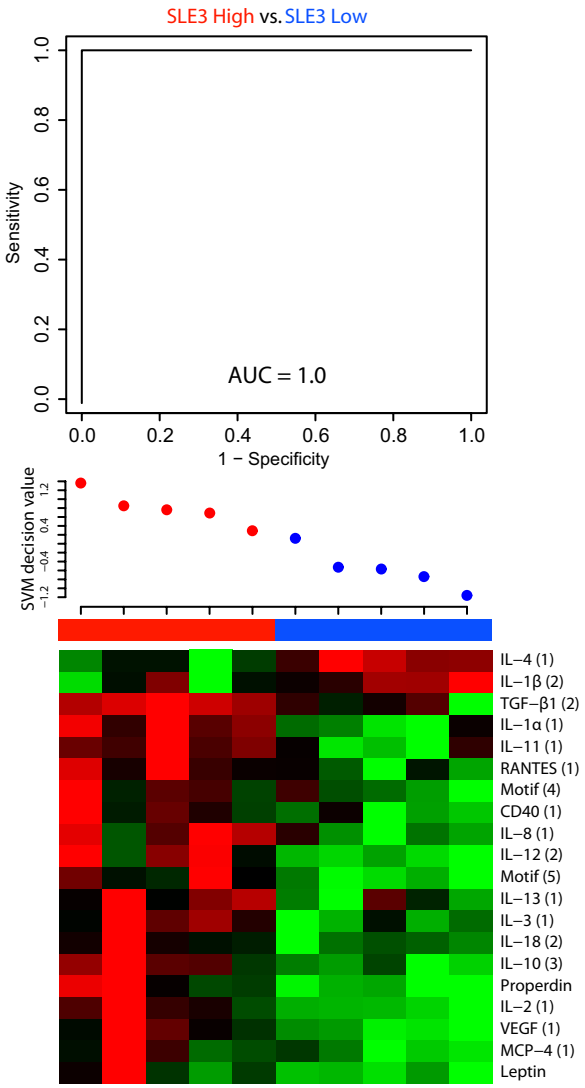

FIG. 7. **Expression profiling of subsets of SLE3 reflecting disease activity, using a 135 recombinant antibody microarray.** Classification of SLE3 subsets, grouped based on disease activity (SLEDAI values); SLE3 low (blue) (mean 13, range 10–16), SLE high (red) (mean 24, range 17–32), based on all 135 antibodies, using a SVM-based leave-one-out cross validation test, expressed in terms of AUC values. The patients are ordered by decreasing decision value as assigned by the SVM classifier (*middle panel*). The 20 signficantly differentially expressed analytes, recognized by 31 antibodies, are shown in a heat map. SSc. Green, down-regulated; red, up-regulated; and black, equal levels.

cesses known to take place as the disease progresses, as mirrored by its unpredictable course and heterogeneous presentation (1–3, 7).

A refined view emerged when the molecular fingerprints of the SLE phenotypic subsets were compared with each other. Then, the data implied a potentially Th1 skewed immune response as the severity of SLE progressed, which is more in line with the common view of a strong inflammatory component in SLE (1–3, 7). The impact of being able to classify SLE into phenotypes reflecting disease severity based on a minimally invasive predictor will be central, because this is vital for therapy selection (1, 5, 11). Currently, no blood-based tests exist for such classification, further indicating the potential of using affinity proteomics for extracting key biological information from complex proteomes. Furthermore, the potentially clinically added value was illustrated by the fact that the candidate biomarker signatures provided better classification than single/combined conventional clinical laboratory variables, *e.g.* ANA, anti-DNA, SLEDAI-2K, C1q, C3, C4, and CRP, in a majority of the cases.

Alternating periods of flare and remission is a typical feature of SLE (1–3). The active phase of the disease often has serious consequences, like tissue and organ damages, and means to predict and monitor a flare would be instrumental for improving patient management (1–3, 11). From a clinical perspective, monitoring the activity of renal involvement in SLE is today mainly dependent on microscopic evaluation of urine, which has been shown to be associated with large methodological shortcomings (10). Other clinical laboratory variables that have been evaluated are *e.g.* auto-antibodies, C1q, C3, and C4, although the performance of these single predictors for monitoring disease activity has varied (60–62). Despite the fact that major efforts have been made to reveal SLE-activity associated markers, ranging from traditional efforts, targeting single analytes, *e.g.* CD40L, to proteomic approaches (57, 58, 60, 63–65), the need for additional information and a refined scenario is significant (6). Here, we have outlined candidate serological biomarker signatures for staging SLE patients based on disease activity that provided better staging than single/combined conventional clinical laboratory variables in a majority of the cases. This identified list of variables was composed of novel markers and some markers that had previously been reported to be indirectly (gene-level) or directly (protein-level) associated with disease activity, such as C3, C4, IL-6, IL-8, IL-10, IL-12, and RANTES (6, 57, 58, 66). In particular interferon-inducible chemokines, of which some were detected here, such as RANTES, IL-6 and IL-8, have frequently been indicated as central players to be associated with disease activity (57, 58, 66, 67). Notably, the first pre-validated three-plexed signature, based on the serum levels of IFN-γ-inducible 10-kDa protein, monocyte chemotactic protein 1, and macrophage inflammatory protein 3β, was recently published (58), outlining the potential of delineating multiplexed serum predictors for staging SLE disease activity.

In order to eliminate the influence of disease severity on our ability to reflect disease activity, we focused on lupus nephritis, the most severe form of SLE, and split this group into 2 cohorts based on disease activity; SLE3-high *versus* SLE3-low. The data showed that we could differentiate the SLE3 samples based on disease activity with high confidence, and the array data was found to provide a better differentiation than single/combined conventional clinical variables in a majority of cases. Again, a mixture of novel and previously re-

ported candidate markers (6, 57, 58, 66) were delineated, further outlining our capability of reflecting disease severity. As compared with when all SLE samples were used, including patients with a broad range of disease severity, the candidate serological biomarker signatures were refined, although several variables were retained, including interferon-inducible chemokines, such as IL-8 and RANTES.

In this pilot study, we have shown that a simple blood sample harbored significant disease-specific information reflecting complex autoimmune connective tissue diseases that could be extracted and interpreted using affinity proteomics. The results could provide new perspective on the nature of SLE, and have outlined several candidate serological biomarker signatures reflecting disease, disease severity and disease activity. The potential of these disease predictors will be validated in larger prospective studies, where also extended array layouts targeting an even wider range of prospective markers will be explored, but suggest novel means for improved clinical management of patients.

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[S] This article contains supplemental Figs. S1 to S6 and Tables S1 and S2.

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Supplemental Table 1 Demographic data of the second, independent SLE cohort included in the study in selected validation experiments.

Parameter	SLE3	Controls
No.	20	20
Gender (female:male)	20:0	20:0
Age	38 (22-70) [a)]	42 (19-66) [a)]
SLEDAI-2K	11 (0-28) [a)]	-

[a)] Median ± range.

Supplemental

Table 2 The different scFv specificities used for the antibody microarrays

Antigen (number of clones)	Antigen (number of clones)
Angiomotin (2)	IL-16 (3)
B cell receptor μ chain (1)	IL-18 (3)
C1 esterase inhibitor (1)	IFN-γ (3)
C1q (1)*	Integrin α-10 (1)
C1s (1)	Integrin α-11 (1)
C3 (2)*	Ku 70/80 (2)
C4 (1)*	LDL (2)
C5 (2)*	Leptin (1)
CD40 (4)	Lewis^{x} (2)
CD40 ligand (1)	Lewis^{y} (1)
Eotaxin (3)	MCP-1 (3)*
Factor B (1)*	MCP-3 (3)
GLP-1 (1)	MCP-4 (2)
GLP-1-R (1)	Mucine-1 (1)
GM-CSF (3)	Peptide motifs (10)
IL-1α (3)	Procathepsin W (1)
IL-1β (3)	Properdin (1)*
IL-1-ra (3)	PSA (1)
IL-2 (3)	Rantes (2)
IL-3 (3)	Sialyl Lewis^{x} (1)
IL-4 (4)*	TGF-β1 (3)
IL-5 (3)*	Tm peptide (1)
IL-6 (4)*	TNF-α (3)
IL-7 (2)	TNF-β (4)*
IL-8 (3)*	Tyrosine-protein kinase BTK (1)
IL-9 (3)	Tyrosine-protein kinase JAK3 (1)
IL-10 (3)*	VEGF (4)*
IL-11 (3)	Choleratoxin subunit B (1) (control)
IL-12 (4)*	FITC (1) (control)
IL-13 (3)*	

* Antibody specificity previously validated by using ELISA, MSD, protein array, blocking/spiking experiments, and/or mass spectrometry.

Supplemental Figure 1

Sample permutations of SVM classifications. The group labels were randomized (permutated) across the samples, and the leave-one-out SVM classification was re-run 20 times using the randomized groups. A) The ROC AUC values obtained are shown for the original sample groups (red circles) versus randomized sample groups (black circles). The wilcoxon p values are also stated. B) The same analysis is performed as in A), but using only significantly ($p<0.05$) differentially expressed analytes) for both the original stratification (red circles) and the åermutated samples (black circles).

Supplemental Figure 2

Validation of antibody array data for SLE versus healthy controls using orthogonal methods. A) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in healthy controls (blue) and SLE (red) determined using an MSD assay and antibody microarrays. The data is shown as boxplots where the median values are indicated (thick line), and the hinges represent the 25th and 75th percentiles, respectively. B) The correlation between antibody array signal intensities for the complement proteins C3, C4, and C1q, and the signal intensities obtained using electroimmuno-, turbidometric-, and/or nephelometric-based assays. The correlations displayed p-values of 0.01, 0.0004 and 6×10^{-7}, respectively.

Supplemental Figure 3

Validation of antibody array data for phenotypic subsets of SLE versus healthy controls using an orthogonal method (MSD assay) and an independent patient cohort. The data is shown as boxplots where the median values are indicated (thick line), and the hinges represent the 25th and 75th percentiles, respectively. A) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in healthy controls (blue) and SLE1 (red) determined using an MSD assay and antibody microarrays. B) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in healthy controls (blue) and SLE2 (red) determined using an MSD assay and antibody microarrays. C) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in healthy controls (blue) and SLE3 (red) determined using an MSD assay and antibody microarrays. D) Comparison of antibody microarray signal intensities for a differentially expressed ($p<0.05$) high-abundant marker, complement protein C3, determined for two independent patient cohorts, set 1 and 2. E) MSD assay signal intensities for selected differentially ($p<0.05$)

expressed cytokines in healthy controls (blue) and SLE3 (red) determined for an independent patient cohort, set 2.

Supplemental Figure 4

Validation of antibody array data for classification of phenotypic subsets of SLE using an orthogonal method (MSD assay). The data is shown as boxplots where the median values are indicated (thick line), and the hinges represent the 25th and 75th percentiles, respectively. A) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in SLE1 (green) and SLE2 (yellow) determined using an MSD assay and antibody microarrays. B) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in SLE2 (yellow) and SLE3 (red) determined using an MSD assay and antibody microarrays. C) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in SLE1 (green) and SLE3 (red) determined using an MSD assay and antibody microarrays.

Supplemental Figure 5

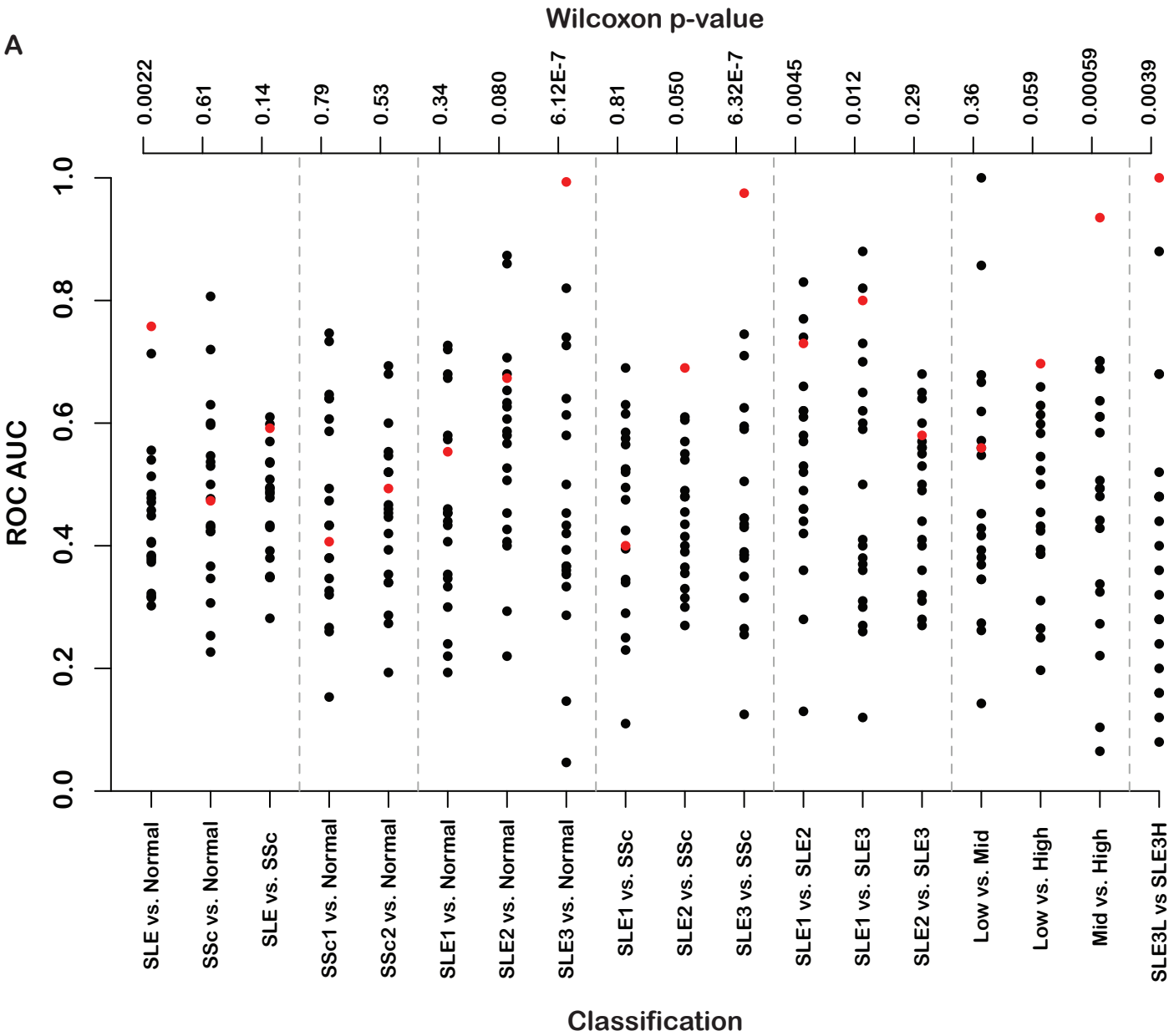
Validation of antibody array data for subsets of SLE reflecting disease activity using orthogonal methods. The data is shown as boxplots where the median values are indicated (thick line), and the hinges represent the 25th and 75th percentiles, respectively. A) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in SLE patients with low disease activity (blue) (based on SLEDAI-2K values) and mid disease activity (red) determined using an MSD assay and antibody microarrays. B) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in SLE patients with mid disease activity (blue) and high disease activity (red) determined using an MSD assay and antibody microarrays. C) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in SLE patients with low disease activity (blue) and high disease activity (red) determined using an MSD assay and antibody microarrays. D) Comparison of signal intensities for selected differentially ($p<0.05$) expressed complement proteins C1q, C3 and C4, in relation to disease activity determined using antibody microarrays versus electroimmuno-, turbidometric-, and/or nephelometric-based assays (denoted as clinical data).

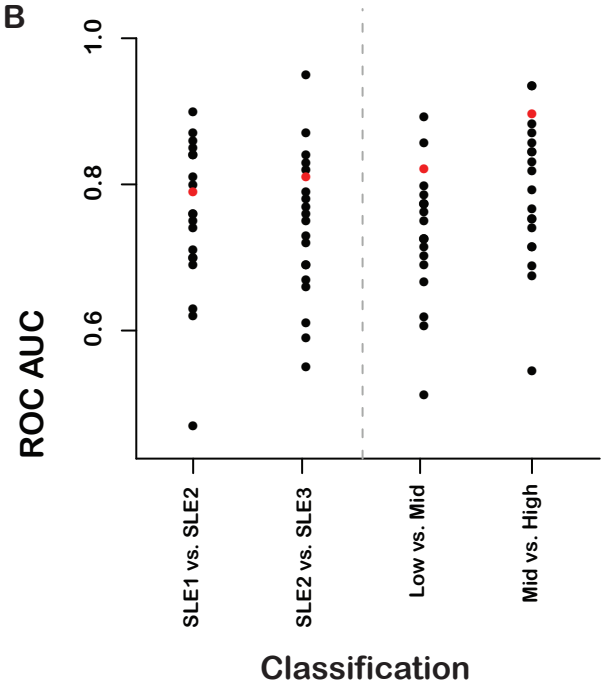
Supplemental Figure 6

Validation of antibody array data for SLE3 patients reflecting disease activity using an orthogonal method (MSD assay) and an independent patient cohort. The data is shown as

boxplots where the median values are indicated (thick line), and the hinges represent the 25th and 75th percentiles, respectively. A) Comparison of signal intensities for selected differentially ($p<0.05$) expressed cytokines in SLE3 patients with low disease activity (blue) (based on SLEDAI-2K values) and high disease activity (red). B) MSD assay signal intensities for selected differentially ($p<0.05$) expressed cytokines in SLE3 pateints with low disease activity (blue) and high disease activity (red) determined for an independent patient cohort, set 2.

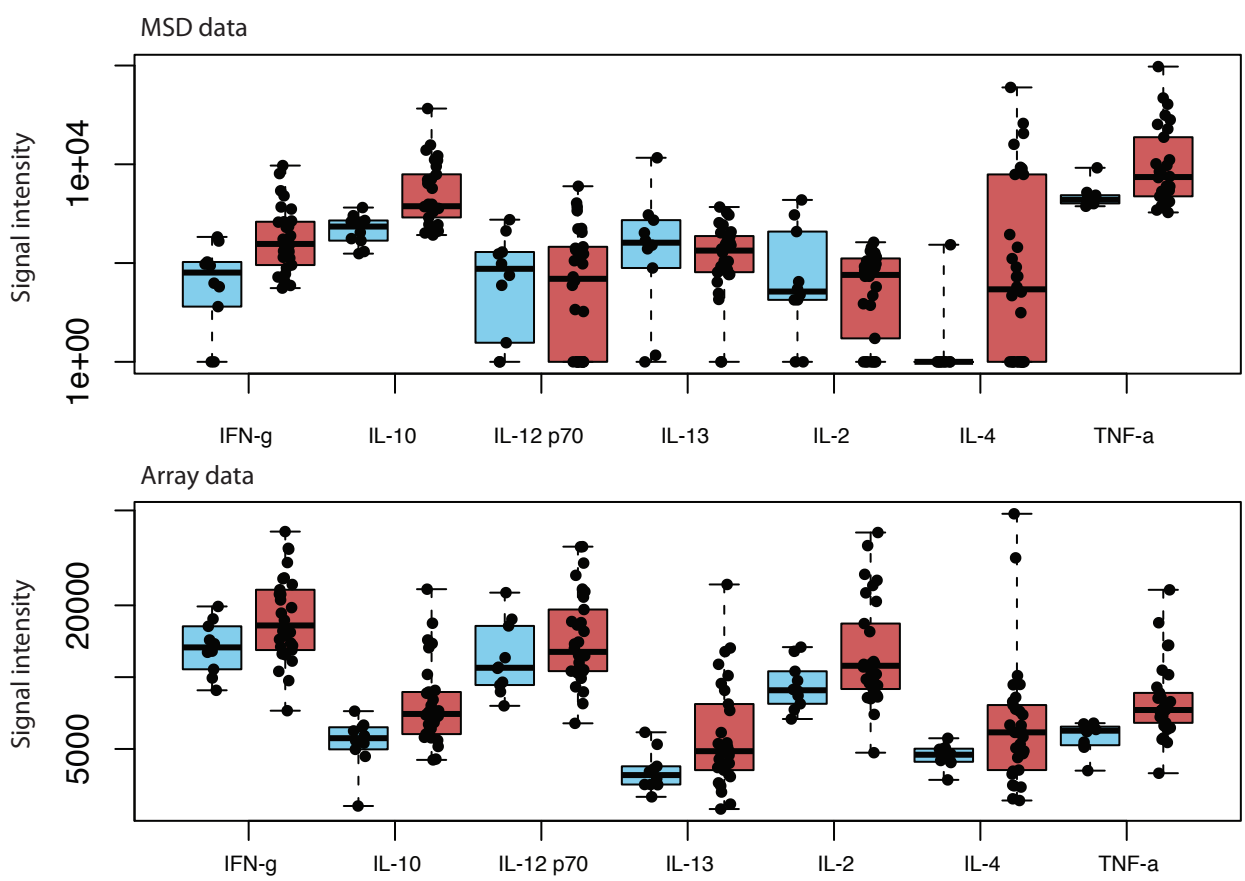
Supplementary figure 1





Supplementary figure 2

A



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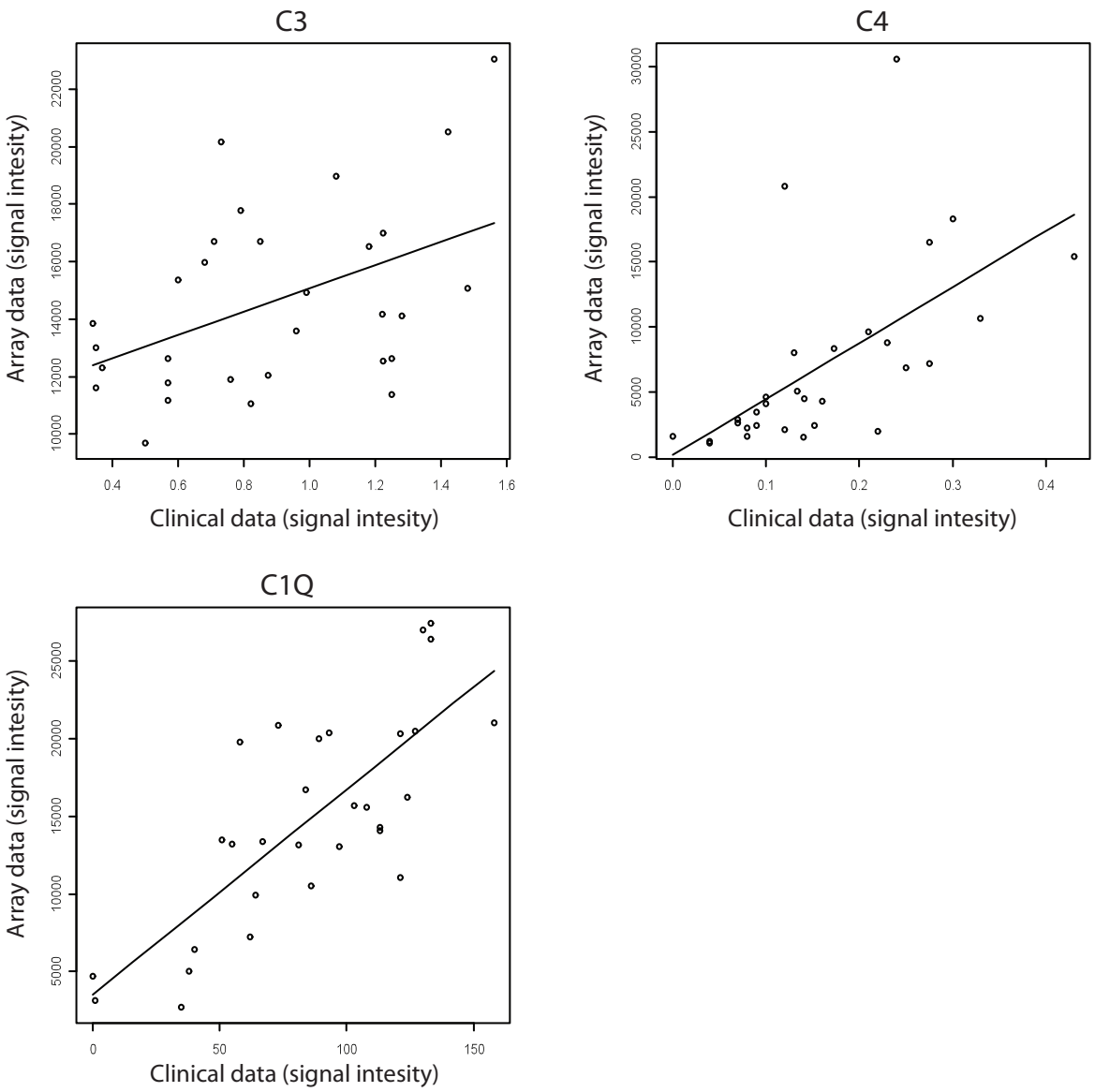

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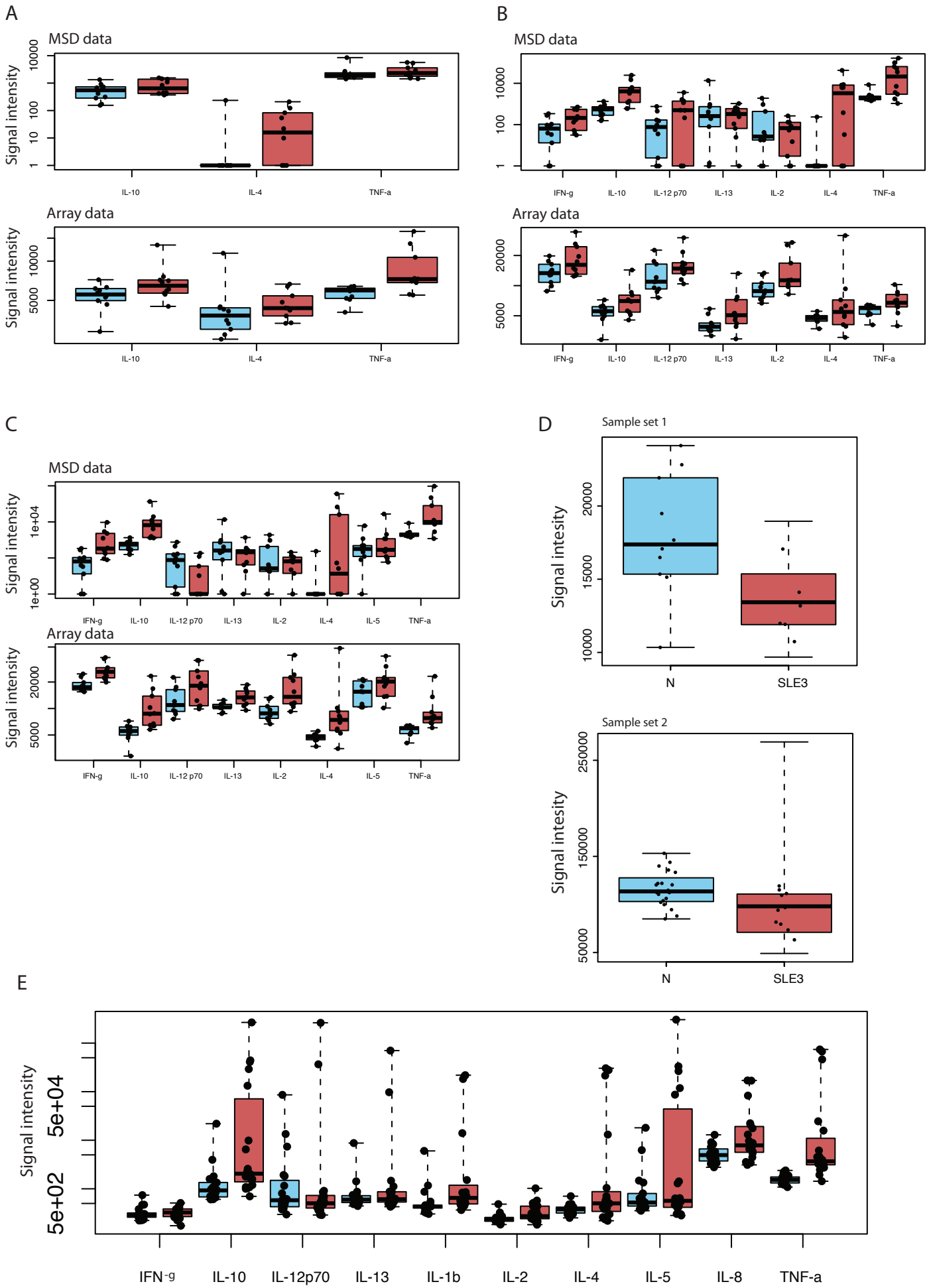

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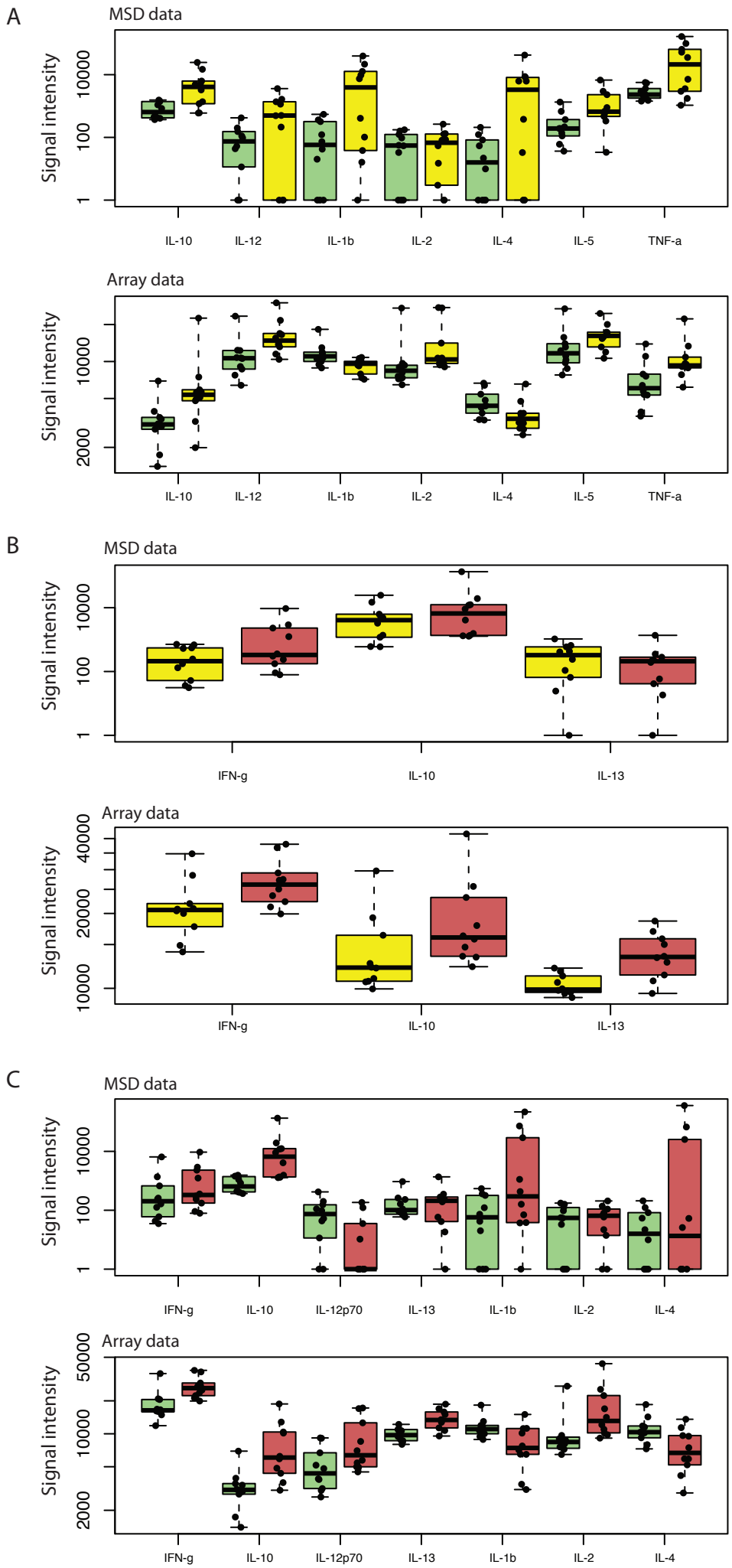

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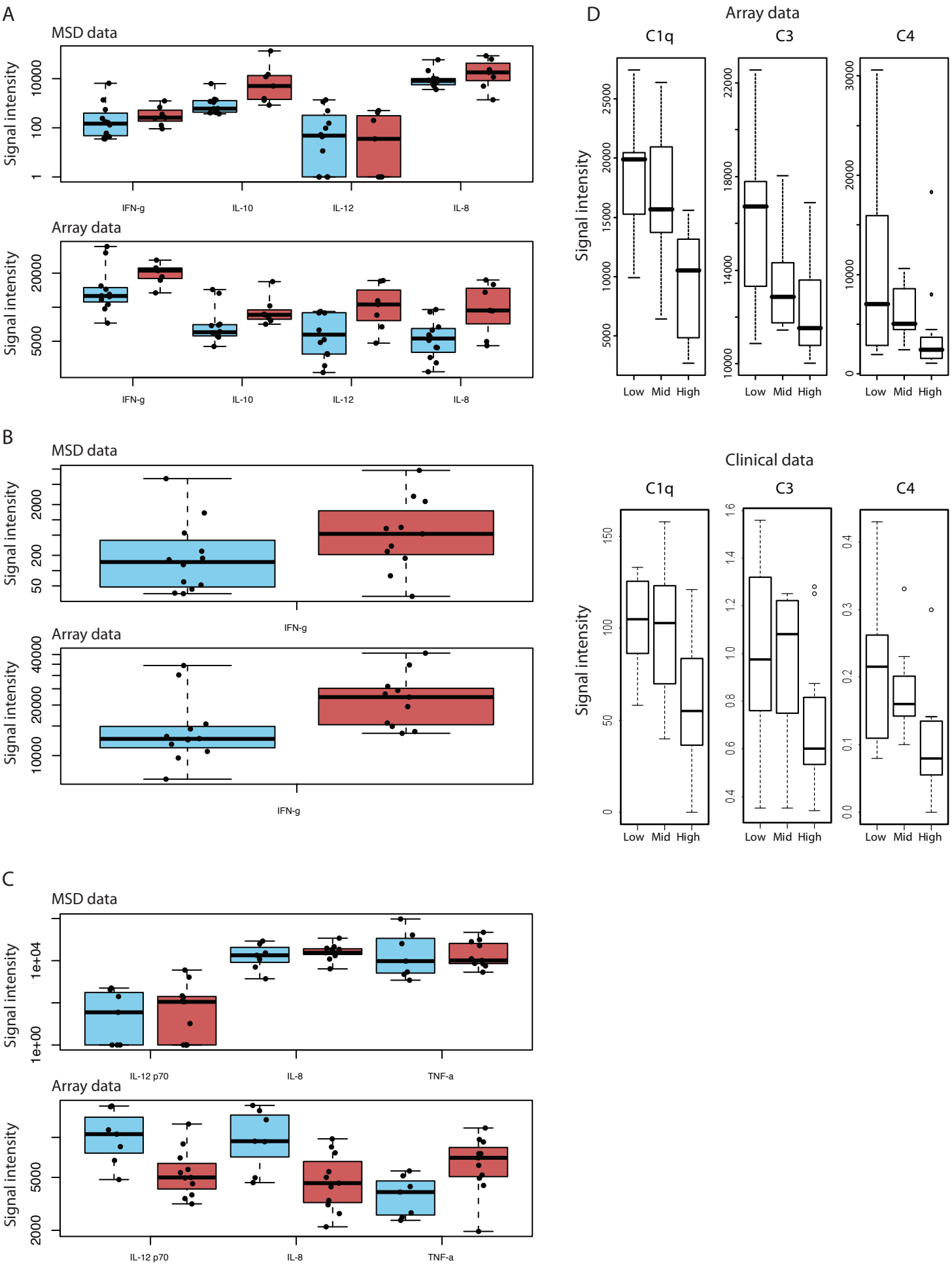
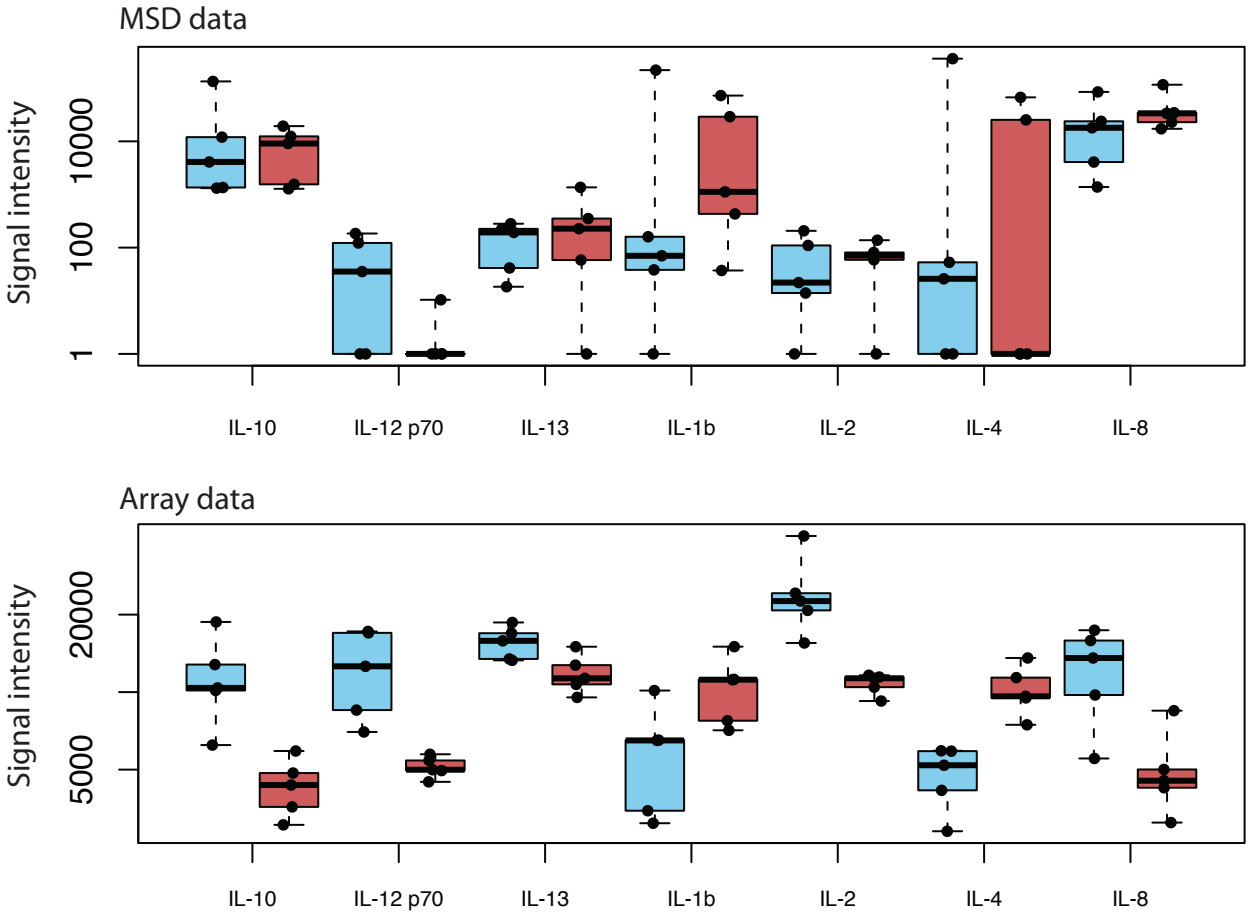
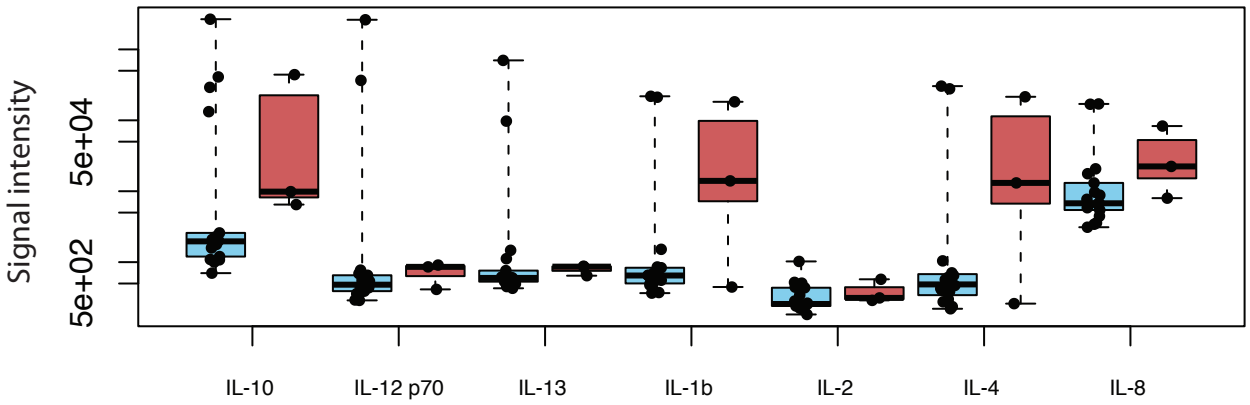


Supplementary figure 6

A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