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IgG and IgM autoantibody differences in discoid and systemic lupus patients

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Abstract

Systemic lupus (SLE) patients with discoid lupus (DLE) were reported to have milder disease. To test this observation, we employed sandwich arrays containing 98 autoantigens to compare autoantibody profiles of SLE subjects without DLE (DLE−SLE+) (N=9), SLE subjects with DLE (DLE+SLE+) (N=10), DLE subjects without SLE (DLE+SLE−) (N=11), and healthy controls (N=11). We validated differentially expressed autoantibodies using immunoassays in DLE−SLE+ (N=18), DLE+SLE+ (N=17), DLE+SLE− (N=23), and healthy subjects (N=22). Arrays showed 15 IgG autoantibodies (ten against nuclear antigens) and four IgM autoantibodies that were differentially expressed (q-value<0.05). DLE−SLE+ subjects had higher IgG autoantibodies against dsDNA, ssDNA, dsRNA, histone H2A and H2B, and SS-A (52 kDa) than all other groups including DLE+SLE+ subjects (p<0.05). Immunoassays measuring anti-dsDNA, -ssDNA, and -SS-A (52 kDa) IgG autoantibodies showed similar trends (p<0.05). Healthy and DLE+SLE −subjects expressed higher IgM autoantibodies against alpha beta crystallin, lipopolysaccharide, heat shock cognate 70, and desmoglein-3 than DLE+SLE+ and DLE−SLE+ subjects. IgG:IgM ratios of autoantibodies against nuclear antigens progressively rose from healthy to DLE−SLE+ subjects. In conclusion, lower IgG autoantibodies against nuclear antigens in DLE+SLE+ versus DLE−SLE+ subjects suggest that DLE indicates lower disease severity. Higher IgM autoantibodies against selected antigens in healthy and DLE+SLE−subjects may be non-pathogenic.

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Conflicts of Interest
The authors state no conflict of interest.

Introduction

Discoid lupus erythematosus (DLE), which is present in up to 20% of systemic lupus erythematosus (SLE) patients (Uramoto *et al.*, 1999), is associated with milder SLE disease activity and lower prevalence of lupus nephritis (Gilliam *et al.*, 1974; Merola *et al.*, 2011; Prystowsky and Gilliam, 1975). 21%–63% of DLE patients have anti-nuclear antibodies (ANAs) (Callen, 1982; Millard and Rowell, 1979; Wallace, 1993), but less than 5% have significant ANA titers (>1:320) seen in SLE patients (Costner, 2008). However, less information is available about autoantibody specificity comparisons between different subsets of DLE and SLE patients.

Proteomic technologies have enhanced our ability to simultaneously and efficiently assess multiple autoantibodies in patient sera. In order to provide comprehensive autoantibody profiles of patients, Robinson *et al* devised miniaturized arrays that contained purified autoantigens. Addition of patient sera and fluorescent secondary antibodies to these arrays facilitates the simultaneous detection of numerous autoantibodies. Moreover, these arrays demonstrate 4–8-fold greater sensitivity in detecting the presence of autoantibodies than enzyme-linked immunosorbant assays (ELISAs) (Robinson *et al.*, 2002).

Autoantigen arrays have generated autoantibody profiles that can discriminate patient groups and give insight into disease progression. In lupus nephritis patients, autoantigen arrays showed that anti-single-stranded DNA (ssDNA), anti-double-stranded DNA (dsDNA), and anti-glomerular antibodies correlated with clinical severity (Li *et al.*, 2005). Our group recently used autoantigen arrays comparing incomplete lupus subjects, who were defined as having one to three American College of Rheumatology (ACR) SLE diagnostic criteria, and SLE subjects. Incomplete lupus subjects showed higher levels of IgM autoantibodies against nuclear antigens and collagens than SLE subjects. This important finding might aid in distinguishing incomplete lupus erythematosus from SLE (Li *et al.*, 2007).

Comparing autoantibody profiles in DLE and SLE subjects may uncover autoantibodies that distinguish these two entities, and shed light in the pathogenesis of DLE. Hence, we conducted a cross-sectional pilot study using autoantigen arrays to compare autoantibody profiles of age- and gender-matched subjects in four groups: 1) SLE subjects without DLE (DLE−SLE+), 2) DLE subjects with SLE (DLE+SLE+), 3) DLE subjects without SLE (DLE+SLE−), and 4) healthy controls. We also performed ELISAs and fluorescent immunoassays to validate differentially expressed autoantibodies in the sera of these subjects. We hypothesized that the levels and types of autoantibodies against nuclear and non-nuclear antigens would discriminate these four groups.

Results

Subject characteristics

The characteristics of age- and gender-matched DLE−SLE+ (N=9), DLE+SLE+ (N=10), DLE+SLE− (N=11), and healthy control (N=11) subjects that were recruited and seroprofiled using autoantigen arrays are displayed in Table 1. The characteristics of the expanded cohort of age- and gender-matched DLE−SLE+ (N=18), DLE+SLE+ (N=17), DLE−SLE+ (N=23), and healthy control (N=22) subjects recruited to confirm array findings via ELISAs and fluorescent immunoassays are displayed in Table 2. All subjects whose sera were evaluated by autoantigen arrays were included in this cohort.

Autoantigen arrays show distinctive patterns of IgG autoantibodies against nuclear antigens in DLE and SLE subjects

The serum levels of 65 IgG autoantibodies meeting minimal net fluorescence intensity (NFI) requirements are presented in a heat map clustered by autoantigen and subject group in Figure 1a. Significance Analysis of Microarrays (SAM) analysis generated a total of 15 IgG autoantibodies that were differentially expressed among the four groups ($q<0.05$) (asterisked in Figure 1a). These included IgG antibodies to C1q, centromere protein-A (CENP-A), desmoglein-3, dsDNA, double-stranded RNA (dsRNA), fibrinogen I-S, histone H1, histone H2A, histone H2B, platelet-derived growth factor receptor (PDGFR) beta, rat glomeruli, SS-A (52 kDa), SS-A (60 kDa), ssDNA, and U1-snRNP-BB'. Ten of these autoantibodies targeted nuclear antigens (anti-CENP-A, -dsDNA, -dsRNA, -histone H1, -histone H2A, -histone H2B, -SS-A (52 kDa), -SSA (60 kDa), -ssDNA, and U1-snRNP-BB') (bracketed in Figure 1a). Autoantibodies targeting dsDNA and SS-A (52 kDa) had the highest levels, with levels of the other autoantibodies being one or two logarithms lower. Both SLE groups showed similar ranges of autoantibody levels. DLE−SLE+ subjects expressed the highest levels of autoantibodies against the aforementioned nuclear antigens ($p<0.05$) except for anti-CENP-A (Figure 1b–k). Most of these autoantibodies showed a stepwise downward progression, starting with DLE−SLE+ subjects and followed by DLE+SLE+, DLE+SLE−, and healthy subjects. Of note, anti-dsDNA, -dsRNA, -histone H2A, -histone H2B, -SS-A (52 kDa), and -ssDNA antibodies were significantly higher in DLE−SLE+ subjects compared with all other groups, most notably DLE+SLE+ subjects ($p<0.05$) (Figure 1b–k).

Other IgG autoantibodies against non-nuclear antigens, including c1q, fibrinogen I-S, and -rat glomeruli, were at the highest levels in DLE−SLE+ subjects ($p<0.05$) (Figure S1a–c). However, the NFIs of these autoantibodies were significantly lower than those of autoantibodies against nuclear antigens highlighted in Figure 1. Antibodies against desmoglein-3, which is important in keratinocyte adhesion, were distinctly elevated in DLE +SLE− subjects versus DLE−SLE+ subjects (Figure S1d).

We also examined for distinctly elevated autoantibodies in DLE+SLE− subjects versus healthy controls. SAM analysis yielded 10 autoantibodies (anti-$\alpha_6\beta_4$ integrin, -β2-microglobulin, -fibrinogen IV, -heparan sulfate proteoglycan (HSPG), -Jo-1, -Matrigel, -proliferating cell nuclear antigen, -PDGFR sR alpha, -SS-A (52 kDa), and -U1-snRNP-A) that were significantly up-regulated in DLE+SLE− subjects ($q<0.05$) (Figure S2a–j). However, levels of all autoantibodies except for anti-HSPG, -Matrigel, and -$\alpha_6\beta_4$ integrin antibodies, in DLE+SLE− subjects were lower than those in DLE−SLE+ subjects, which represented disease controls.

Orthogonal platforms confirm array trends in IgG autoantibodies against nuclear antigens in DLE and SLE subjects

We performed ELISAs and fluorescent immunoassays using an independent cohort of subjects to verify autoantigen array trends in IgG autoantibodies against nuclear antigens. Once again, anti-dsDNA, -ssDNA, and -SS-A (52 kDa) IgG antibodies were significantly higher in DLE−SLE+ subjects than the other three groups, including DLE+SLE+ subjects ($p<0.005$) (Figure 2a–c). DLE−SLE+ and DLE+SLE+ subjects expressed similarly elevated amounts of anti-SS-A (60 kDa), -U1-snRNP-BB', -histones, and ANA IgG antibodies compared with DLE+SLE− and healthy subjects ($p<0.05$) (Figure 2d–g). Anti-dsRNA, -histone H1, -histone H2A, -histone H2B, and SS-B IgG antibodies were not significantly different among the four groups (data not shown). Comparison of autoantigen array and immunoassay values for each subject showed strong correlation for anti-dsDNA (Spearman's $r=0.72$, $p<0.0001$), -SS-A (52 kDa) (Spearman's $r=0.38$, $p=0.01$), -SS-A (60 kDa) (Spearman's $r=0.59$, $p<0.0001$), and -ssDNA (Spearman's $r=0.78$, $p<0.0001$) (Figure 2h–k).

In contrast, immunoassays measuring IgG autoantibodies against selected skin antigens did not reflect similar trends seen in the autoantigen arrays. We performed ELISAs measuring IgG autoantibodies against the epidermal-dermal junction proteins $\alpha_6\beta_4$ integrin and HSPG, which were up-regulated on the arrays in DLE+SLE− subjects versus healthy controls. DLE+SLE− and healthy subjects expressed similar levels of IgG autoantibodies against $\alpha_6\beta_4$ integrin and HSPG. In addition, ELISAs evaluating anti-desmoglein-3 IgG antibodies, which were down-regulated in DLE−SLE+ subjects in the arrays, showed no distinct differences (data not shown).

Decreased IgM autoantibodies against selected antigens were seen in SLE subjects

A heat map summarizing serum levels of IgM antibodies against 85 autoantigens, which met NFI requirements, is presented in Figure 3a. SAM analysis identified four autoantibodies (anti-alpha B crystallin, -desmoglein 3, -heat shock cognate 70 (Hsc70), and -lipopolysaccharide (LPS)) that were differentially expressed in the four groups (asterisked in Figure 3a). IgM autoantibodies against alpha B crystallin and LPS were highest in healthy controls, followed by DLE+SLE−, DLE+SLE+, and DLE−SLE+ subjects (Figure 3b–c). IgM autoantibodies against Hsc70 and desmoglein-3 were elevated in healthy and DLE+SLE− subjects than in the other groups (Figure 3d–e). SAM analysis did not yield any significantly up-regulated IgM autoantibodies in DLE+SLE− subjects versus healthy controls.

Increased IgG:IgM ratios in autoantibodies against predominantly nuclear antigens are seen in DLE−SLE+ subjects

Ratios of IgG and IgM NFIs were calculated for each differentially expressed IgG and IgM autoantibody among the four groups. For all 15 differentially expressed IgG autoantibodies, the highest IgG:IgM ratios were found in the DLE−SLE+ group, with statistical significance being attained with anti-dsDNA, -dsRNA, -fibrinogen I-S, -histone H2A and H2B, -rat glomeruli, and – SS-A (60 kDa) antibodies ($p<0.05$) (Figure 4a–g). Furthermore, in this group of autoantibodies, a stepwise decrease in IgG:IgM ratio was noted from DLE−SLE+

subjects to DLE+SLE+ subjects and finally both non-SLE groups. IgG:IgM ratios in the four differentially expressed IgM autoantibodies failed to show significant patterns in the four groups.

Discussion

This study generated comprehensive autoantibody profiles of DLE and SLE subjects using autoantigen arrays. Comparison of these profiles showed that autoantibodies against various nuclear antigens can stratify DLE and SLE subjects. Six autoantibodies against dsDNA, dsRNA, histone H2A, histone H2B, SS-A (52 kDa), and ssDNA showed distinctively higher levels in DLE−SLE+ subjects versus all other groups, most notably DLE+SLE+ subjects. Immunoassays measuring IgG autoantibodies against dsDNA, ssDNA, and SS-A (52 kDa) displayed this similar trend. Lower levels of IgG autoantibodies against nuclear antigens in DLE+SLE+ subjects compared with DLE−SLE+ subjects imply that DLE is a phenotypic marker associated with milder systemic disease. A possible explanation for the decrease in IgG autoantibodies against nuclear antigens in DLE+SLE+ vs. DLE−SLE+ subjects is that the skin may serve as a repository for autoantibody deposition in DLE patients and decrease the number of circulating autoantibodies that could inflict peripheral organ damage. Potential targets of autoantibodies in DLE skin include nuclear antigens from keratinocytes. Cultured keratinocytes treated with ultraviolet light have undergone apoptosis, exposed nuclear antigens, and shown increased binding to various autoantibodies such as those targeting SS-A and SS-B (Furukawa *et al.*, 1999).

Discoid lesions have been previously associated with lower overall disease severity, specifically reduced renal involvement and rates of positive ANAs (Callen, 1982; Gilliam *et al.*, 1974; Prystowsky and Gilliam, 1975). A comparison of our DLE−SLE+ and DLE+SLE+ subjects reveals that a smaller portion of DLE+SLE+ subjects had renal and neurological disease, and serositis. A previous study of 201 Puerto Ricans with SLE showed higher percentages of positive tests for anti-Sm and RNP antibodies in DLE−SLE+ subjects than in DLE+SLE+ subjects, but actual autoantibody levels were not reported (Vila *et al.*, 2006).

Our data showing elevated IgG autoantibodies against nuclear antigens in SLE subjects are consistent with previous observations in SLE patients (Adu *et al.*, 1981). The DLE−SLE+ group, which had the highest percentage of renal disease, expressed the highest autoantibodies against dsDNA and histone proteins, which are associated with lupus nephritis (Adu *et al.*, 1981; Cortes-Hernandez *et al.*, 2004). These autoantibodies correlate with disease activity in SLE patients (Cortes-Hernandez *et al.*, 2004). Anti-ssDNA, anti-SS-A, and anti-RNP antibodies are found in at least half of all SLE patients (Ignat *et al.*, 2003; Li *et al.*, 2010b). Apoptotic activity in the kidney can lead to enhanced release of nucleosomes containing DNA and histones, making them prime targets for autoantibodies (Kalaaji *et al.*, 2006). Glomerular deposits of histones and nucleosomes have been observed in human lupus nephritis kidneys, where immune complexes accumulate and trigger lupus (van Bruggen *et al.*, 1997).

The predominant autoantibody in DLE patients remains unknown. Various autoantibodies against nuclear antigens including dsDNA, Smith, SS-A, SS-B, and ssDNA (Lee *et al.*, 1994; Provost *et al.*, 1985; Wallace, 1993) have been previously tested, but only low-titer anti-Ro (60 kDa) antibodies have been found in most DLE patients (Lee *et al.*, 1994). Our autoantigen arrays provided a comprehensive scan of autoantibodies in DLE sera by screening for 98 autoantibodies involved in various autoimmune systemic and/or cutaneous diseases. Compared with healthy controls, DLE+SLE− subjects had ten up-regulated autoantibodies, which included three against nuclear antigens (Jo-1, U1-snRNP-A', SS-A (52 kDa)) and two against epidermal-dermal junction proteins (HSPG, $\alpha_6\beta_4$ integrin). The three antibodies against nuclear antigens that were elevated in DLE+SLE− subjects were markedly lower than in DLE−SLE+ subjects, thus decreasing their specificity in DLE. ELISAs measuring autoantibodies against HSPG and $\alpha_6\beta_4$ integrin failed to verify the autoantigen array results. This was likely due to either relatively low NFIs (anti-$\alpha_6\beta_4$ integrin antibody) or fold-change differences (anti-HSPG antibody) in the autoantigen arrays. Based on these findings and the results of decreased autoantibodies against nuclear antigens in DLE+SLE+ versus DLE−SLE+ subjects, we hypothesize that autoantibodies distinctly elevated in DLE patients may be found in the skin rather than in the sera. Further studies isolating antibodies deposited in DLE skin will be pursued.

Four distinct IgM autoantibodies identified as differentially expressed among the four groups by SAM analysis tended to have lower levels in the two SLE groups (DLE+SLE+ and DLE−SLE+) than the two non-SLE groups (DLE+SLE− and healthy). Moreover, multiple other IgM autoantibodies, which were in the same clusters as these aforementioned antibodies, expressed a similar pattern among the groups. These findings are consistent with our previous autoantigen array findings that IgM autoantibodies were higher in incomplete lupus erythematosus subjects (Li *et al.*, 2007). We hypothesize that because the vast majority of DLE patients do not progress to SLE, specific IgM autoantibodies may either halt or fail to induce systemic progression. Lupus-prone MRL-lpr mice that could not secrete IgM antibodies enhanced production of IgG autoantibodies against dsDNA and histones compared with wild-type MRL-lpr mice. Moreover, these mice had more severe glomerulonephritis and shorter life span than their normal counterparts (Boes *et al.*, 2000).

We also observed a marked increase in IgG:IgM ratios in autoantibodies that mainly targeted nuclear antigens (e.g. dsDNA, dsRNA, ssDNA) in DLE−SLE+ subjects versus the other subjects, especially DLE+SLE+ subjects. This trend mirrored that of the same IgG autoantibodies in the four groups. This may relate to the ability of IgG autoantibodies to elicit FcR-dependent pathogenic cascades in peripheral organs. Additionally, it may reflect a more robust class switching drive (with attendant somatic mutation) (Shlomchik *et al.*, 1990) among the DLE−SLE+ subjects, possibly because of their genetic makeup, the nature of autoantigens in these patients, or the antigenic or inflammatory milieu within their germinal centers. Indeed, it would be interesting to examine if DLE−SLE+ subjects had more vibrant germinal center responses. Finally, this may relate to the potentially protective role of IgM autoantibodies, as previously demonstrated in IgM-deficient MRL-lpr mice (Boes *et al.*, 2000). This characteristic is likely limited to selected IgM autoantibodies. Injection of anti-dsDNA IgM antibodies, but not anti-Sm and anti-phospholipid IgM antibodies, in MRL-lpr mice alleviated lupus nephritis. This may be due to decreased macrophage infiltration and cytokine production, and more efficient clearance of apoptotic debris (Jiang *et al.*, 2011). The relative decrease in IgG:IgM autoantibodies against these antigens in DLE+SLE+ versus DLE−SLE+ subjects may also explain the lower prevalence of lupus nephritis in DLE patients (Merola *et al.*, 2011; Prystowsky and Gilliam, 1975). A prospective study following autoantibody profiles of these subjects and mechanistic studies in MRL-lpr mice would further clarify the relative roles of IgG and IgM autoantibodies in DLE and SLE.

Limitations of our study include small sample size, selection bias, cross-sectional nature, and potential for falsely significant autoantibodies. Despite our small sample size, we still found significant differences in autoantibodies against nuclear antigens between DLE and SLE subjects. While subjects were mostly selected from one tertiary referral center, we were able to recruit DLE and SLE subjects with a wide range of disease activity. A larger multi-center study sampling the sera of DLE and SLE subjects is being planned to verify our findings. In addition, sera from patients were collected from only one visit. Comparing autoantibody profiles at multiple visits from the same patients in a future prospective study will help identify those whose levels correlate with disease activity. While sampling of multiple autoantibodies can result in identifying autoantibodies that are falsely significantly different, a false discovery rate of 5% (q-value) on SAM analysis was established to minimize this error, and immunoassays were performed to verify autoantigen array results.

We have demonstrated that there are distinctive patterns of IgG and IgM autoantibodies that may distinguish subsets of DLE and SLE subjects. The vast majority of differentially expressed IgG autoantibodies targeted nuclear antigens. Specifically, DLE−SLE+ subjects expressed the highest level of autoantibodies against dsDNA, dsRNA, histone H2A, histone H2B, ssDNA, and SS-A (52 kDa) on autoantigen arrays and immunoassays. The down-regulation of these autoantibodies in DLE+SLE+ versus DLE−SLE+ subjects supports previous clinical findings that DLE patients have milder systemic disease. Downward trends in selected IgM autoantibodies against alpha B crystallin, desmoglein 3, Hsc70, and LPS were noted in both SLE groups. DLE−SLE+ subjects had the highest IgG:IgM ratios against autoantibodies against mostly nuclear antigens. We have hypothesized non-pathogenic roles for specific IgM autoantibodies, which would require confirmation in larger human sera studies. Future investigation into their function in murine lupus models could provide new insights into combating SLE.

Materials and Methods

Subjects

This was a cross-sectional pilot study comparing serum autoantibody values from age-, and gender-matched DLE, SLE, and healthy control subjects who presented to outpatient dermatology and rheumatology clinics at the University of Texas Southwestern Medical Center and Parkland Health and Hospital System in Dallas, TX. All subjects were recruited from July 2003 to January 2011. All subjects consented by written agreement to inclusion in this study, which was approved by the University of Texas Southwestern Medical Center Institutional Review Board. The study protocol and informed consent were in compliance with Declaration of Helsinki Principles. 18 DLE−SLE+ subjects, 17 DLE+SLE+ subjects, 23 DLE+SLE− subjects, and 22 healthy controls were recruited and enrolled into the Dallas

Regional Autoimmune Disease Registry and/or University of Texas Southwestern Cutaneous Lupus Registry. The inclusion criteria for all subjects were the ability to give written informed consent, and age above 18 years. Subjects were excluded if they had drug-induced SLE or DLE. Healthy controls were excluded if they had a history of an autoimmune disease. DLE−SLE+ subjects were defined as those meeting at least four ACR SLE diagnostic criteria (Tan *et al.*, 1982) without having a history of DLE. DLE+SLE+ subjects fulfilled at least four ACR SLE diagnostic criteria including DLE. The diagnosis of DLE was based on clinicopathologic correlation. While carrying the DLE diagnosis, DLE +SLE− subjects had less than four ACR SLE diagnostic criteria.

Data collection

At the time of enrollment, study subjects provided information on demographics, past medical histories, and current treatments. Cutaneous and systemic disease severities were assessed using the Cutaneous Lupus Disease Activity and Severity Index (Albrecht *et al.*, 2005) and Systemic Lupus Erythematosus Disease Activity Index (Bombardier *et al.*, 1992), respectively. Other information such as laboratory values and biopsy reports were obtained by medical chart review.

Blood collection

Approximately 5 mL of blood was drawn in serum separator tubes from each subject. Sera was collected after centrifugation of blood samples at 3000 rpm at room temperature for 10 minutes, and stored in aliquots at −80°C.

Autoantigen arrays

Autoantigen arrays were designed by plating recombinant or purified proteins from 98 antigens, which were associated with either autoimmune cutaneous diseases (Table S1), or systemic diseases, as previously described (Li *et al.*, 2011). We prepared antigens, coated slides, incubated patient serum and secondary fluorescently-conjugated antibodies, as previously described (Li *et al.*, 2005; Li *et al.*, 2010a). A Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA) detected fluorescent signals and generated images for analysis (Li *et al.*, 2007). NFIs for IgG autoantibodies were normalized by dividing background-adjusted values with those from anti-human IgG in the array and multiplying the ratio by 1000. We excluded IgG and IgM autoantibodies whose levels were less than 1% of the highest NFI for all subject samples, as these were regarded as noise.

Immunoassays

ELISAs were performed to measure IgG autoantibodies of interest identified from the arrays with commercially available kits (ANAs (INOVA Diagnostics, Inc., San Diego, CA), anti-dsDNA antibodies, anti-ssDNA, anti-histones antibodies (ORGENTEC Diagnostika, Mainz-Germany), and anti-desmoglein-3 antibodies (MBL International, Woburn, MA)). Concentrations were extrapolated from a standard curve. Established ELISA protocols (Shi *et al.*, 2002) were used to measure IgG autoantibodies against U1-snRNP-BB’ (Surmodics, Eden Prairie, MN), histone H1 (Roche, Indianapolis, IN), H2A, H2B proteins (New England BioLabs, Ipswich, MA), dsRNA/polyinosinic-polycytidylic RNA (Sigma-Aldrich, St. Louis, MO), HSPG (Sigma-Aldrich), and $\alpha_6\beta_4$ integrin (R&D Systems, Minneapolis, MN) and IgM autoantibodies against LPS (Sigma-Aldrich). OD_{450} was measured by an Elx800 microplate reader (Biotek Instruments, Winooski, VT). Fluorescent immunoassays were performed to measure anti-SS-A (52 kDa), -SS-A (60 kDa), and SS-B IgG antibodies using QUANTA Plex™ (Luminex®) kits (INOVA Diagnostics, Inc.).

Statistical analysis

Sample size was not calculated because of the pilot study design. SAM analysis (http://www-stat.stanford.edu/~tibs/SAM/) was used to determine autoantibodies with statistically significant differences among groups. Heat maps were generated, and row-wise clustering analysis was performed using Cluster and Treeview software (http://rana.lbl.gov/EisenSoftware.htm). Subject characteristics were compared using either one-way analysis of variance (ANOVA) tests (continuous variables), Student's t-tests (continuous variables), or Fisher's exact tests (categorical variables). For the autoantibodies that were identified to be differentially expressed by SAM analysis, secondary analyses using one-way ANOVA with Tukey's honestly significant difference (HSD) tests were conducted to assess pairwise differences among disease groups. The correlation between autoantigen array and immunoassay values for selected autoantibodies in subject sera was assessed by calculating Spearman's correlation coefficient and accompanying p-values. Statistical significance was declared for p-values (ANOVA, Tukey's test) and q-values, which are defined as the lowest false discovery rate at which the autoantibodies are called significant by SAM analysis (Tusher *et al.*, 2001), less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACR	American College of Rheumatology
ANA	anti-nuclear antibody
ANOVA	analysis of variance
CENP-A	centromere protein-A
DLE	discoid lupus erythematosus
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA

ELISAs	enzyme-linked immunosorbant assays
Hsc70	heat shock cognate 70
HSD	honestly significant difference
HSPG	heparan sulfate proteoglycan
LPS	lipopolysaccharide
PDGFR	platelet-derived growth factor receptor
SAM	Significance Analysis of Microarrays
SLE	systemic lupus erythematosus
ssDNA	single-stranded DNA

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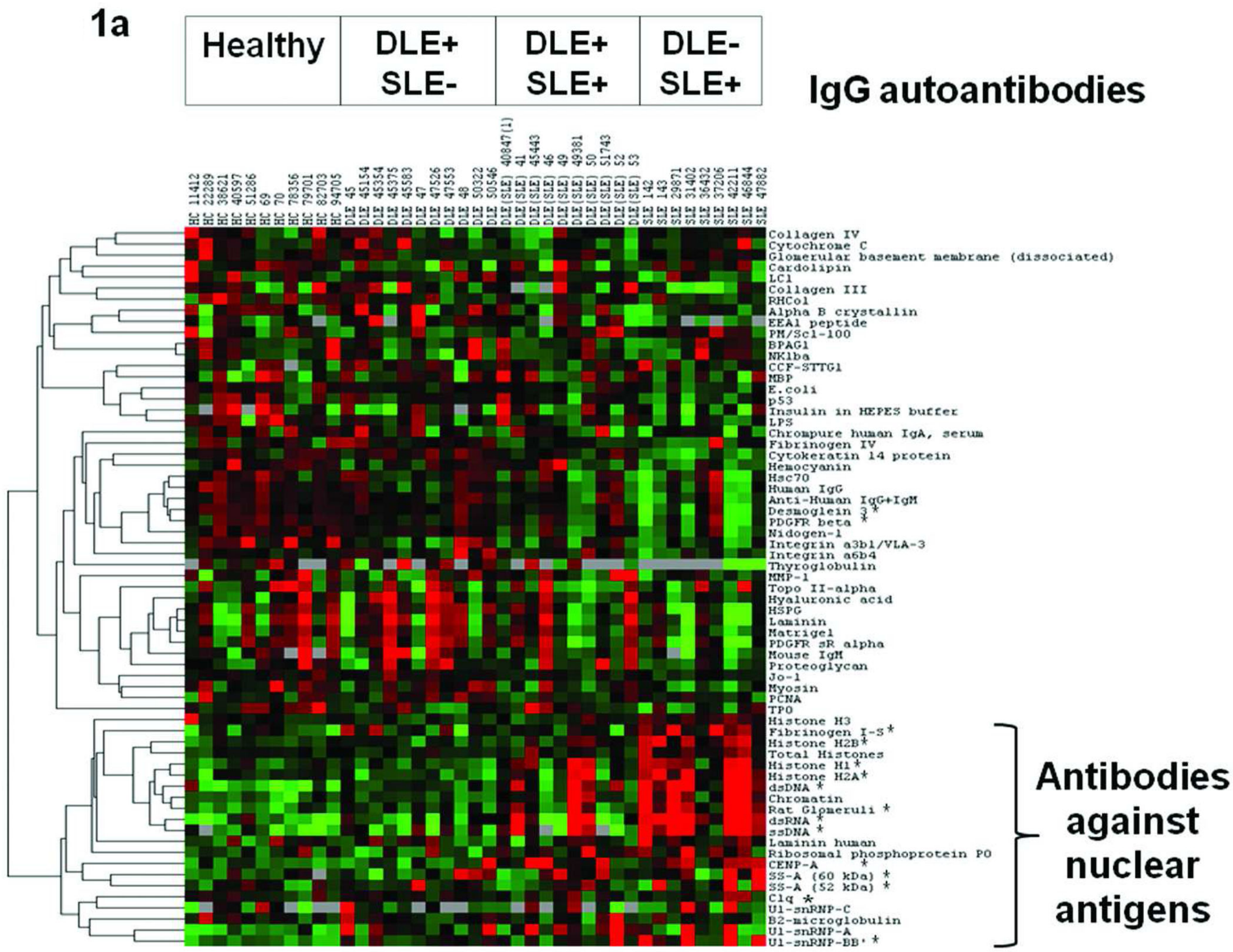

1a
Healthy
DLE+ SLE-
DLE+ SLE+
DLE- SLE+
IgG autoantibodies
HC 11412
HC 22289
HC 38621
HC 40597
HC 51286
HC 69
HC 70
HC 78356
HC 79701
HC 82703
HC 94705
DLE 45
DLE 45154
DLE 45354
DLE 45375
DLE 45583
DLE 47
DLE 47526
DLE 47553
DLE 48
DLE 50322
DLE 50546
DLE (SLE) 40847(1)
DLE (SLE) 41
DLE (SLE) 45443
DLE (SLE) 46
DLE (SLE) 49
DLE (SLE) 49381
DLE (SLE) 50
DLE (SLE) 51743
DLE (SLE) 52
DLE (SLE) 53
SLE 142
SLE 143
SLE 29871
SLE 31402
SLE 36432
SLE 37206
SLE 42211
SLE 46844
SLE 47882
Collagen IV
Cytochrome C
Glomerular basement membrane (dissociated)
Cardolipin
LC1
Collagen III
RHCol
Alpha B crystallin
EEA1 peptide
PM/Scl-100
BPAG1
NK1ba
CCF-STTG1
MBP
E.coli
p53
Insulin in HEPES buffer
LPS
Chrompure human IgA, serum
Fibrinogen IV
Cytokeratin 14 protein
Hemocyanin
Hsc70
Human IgG
Anti-Human IgG+IgM
Desmoglein 3 *
PDGFR beta *
Nidogen-1
Integrin a3b1/VLA-3
Integrin a6b4
Thyroglobulin
MMP-1
Topo II-alpha
Hyaluronic acid
HSPG
Laminin
Matrigel
PDGFR sR alpha
Mouse IgM
Proteoglycan
Jo-1
Myosin
PCNA
TPO
Histone H3
Fibrinogen I-S *
Histone H2B *
Total Histones
Histone H1 *
Histone H2A *
dsDNA *
Chromatin
Rat Glomeruli *
dsRNA *
ssDNA *
Laminin human
Ribosomal phosphoprotein P0
CENP-A *
SS-A (60 kDa) *
SS-A (52 kDa) *
C1q *
U1-snRNP-C
B2-microglobulin
U1-snRNP-A
U1-snRNP-BB' *
Antibodies against nuclear antigens

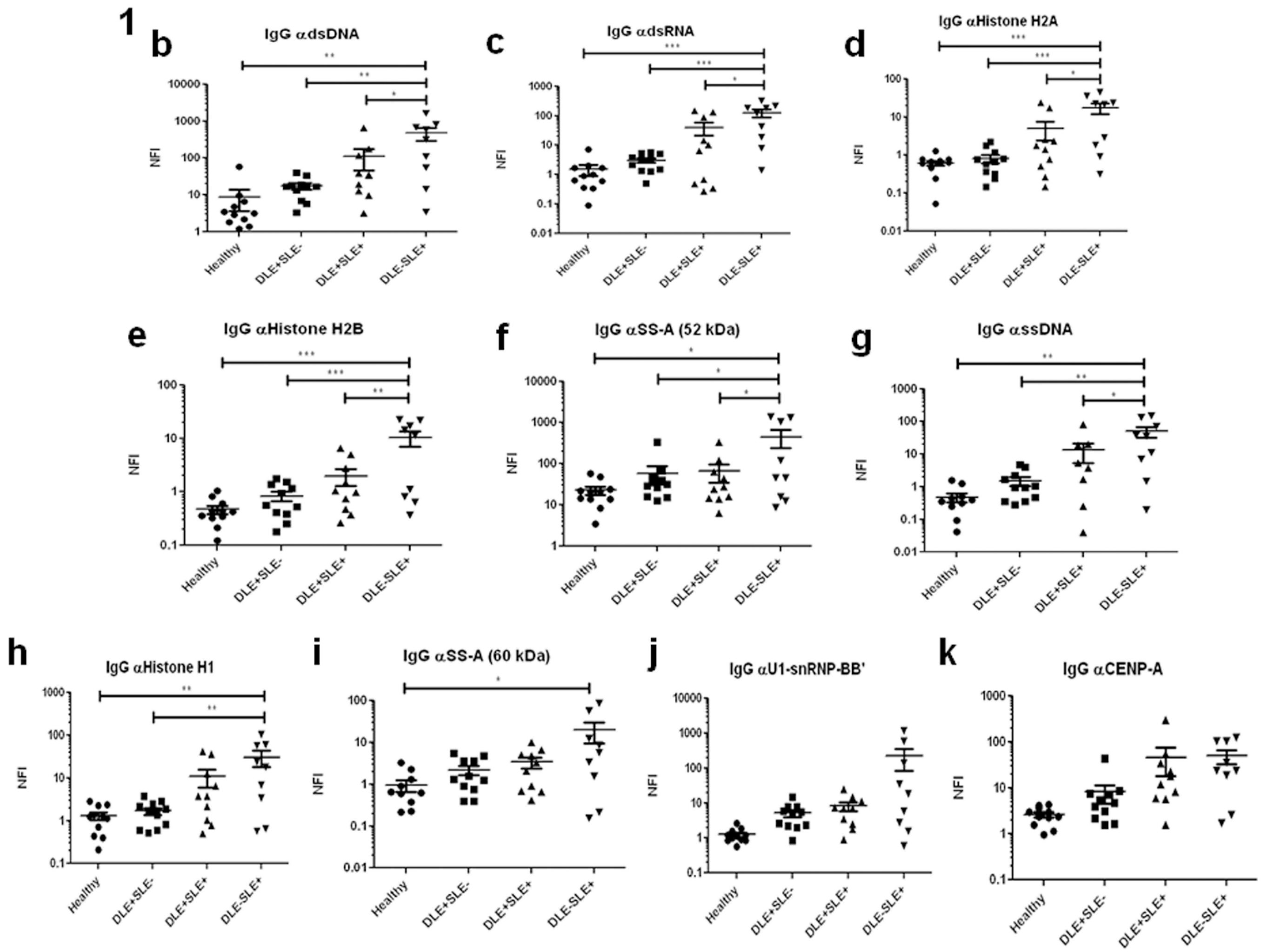
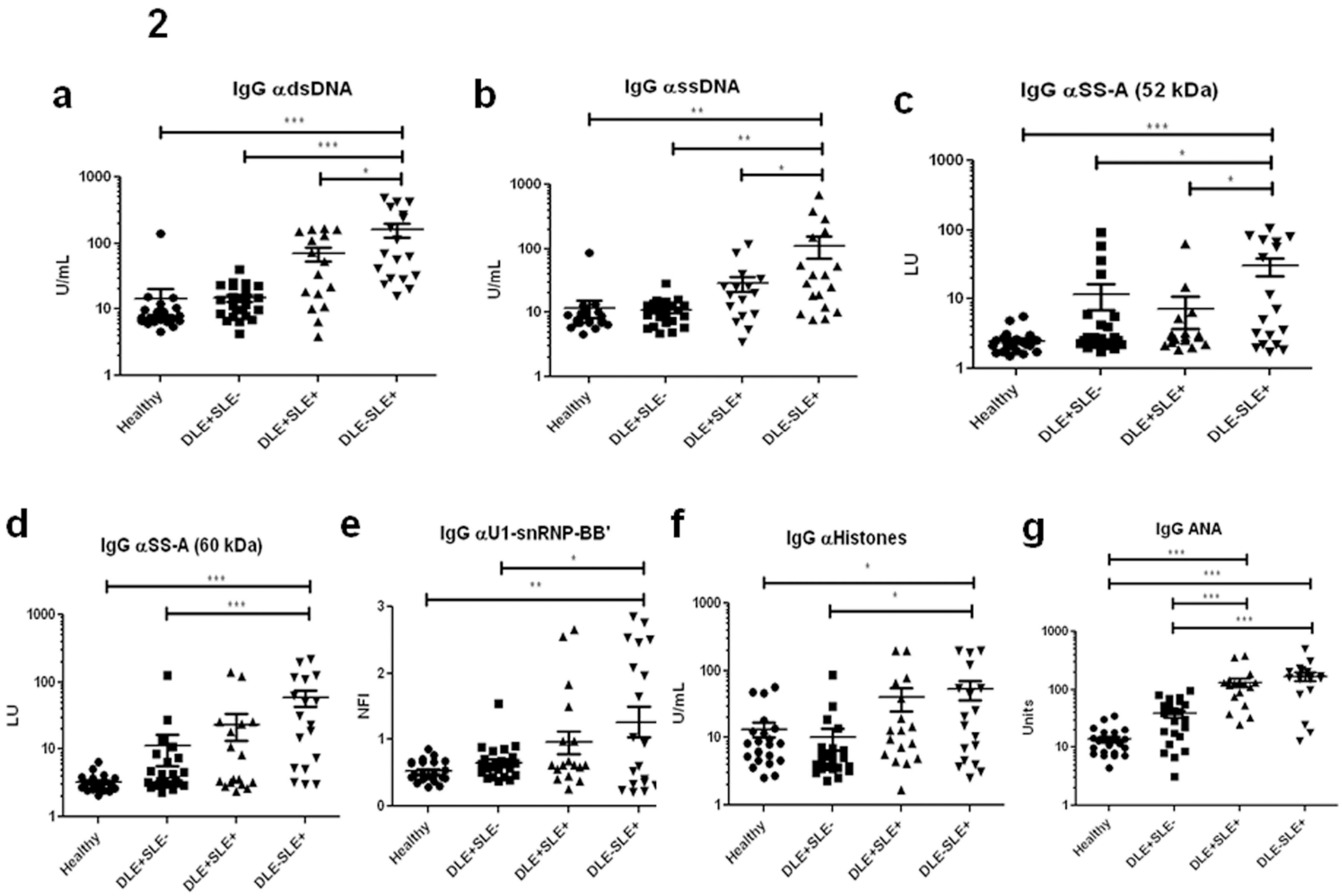


Figure 1. IgG autoantibody levels in DLE and SLE sera as determined by autoantigen arrays

(**a**) We generated a heat map summarizing IgG reactivities in the four groups. Green, black, and red represent NFIs below, close to, and above the mean, respectively. SAM analysis identified differentially expressed autoantibodies (*: q<0.05). The lower right bracket spans autoantibodies targeting multiple nuclear antigens. (**b–k**) For each group, we plotted NFIs for differentially expressed IgG autoantibodies against dsDNA (**b**), dsRNA (**c**), histone H2A (**d**), histone H2B (**e**), SS-A (52 kDa) (**f**), ssDNA (**g**), histone H1 (**h**), SS-A (60 kDa) (**i**), U1-snRNP-BB' (**j**), and CENP-A (**k**). We performed secondary analyses using one-way ANOVA with Tukey's HSD test for multiple comparisons. *: p<0.05, **: p<0.005, ***: p<0.0005.


2
a
IgG αdsDNA
U/mL
Healthy
DLE+SLE-
DLE+SLE+
DLE-SLE+
b
IgG αssDNA
c
IgG αSS-A (52 kDa)
LU
d
IgG αSS-A (60 kDa)
e
IgG αU1-snRNP-BB'
NFI
f
IgG αHistones
g
IgG ANA
Units

2

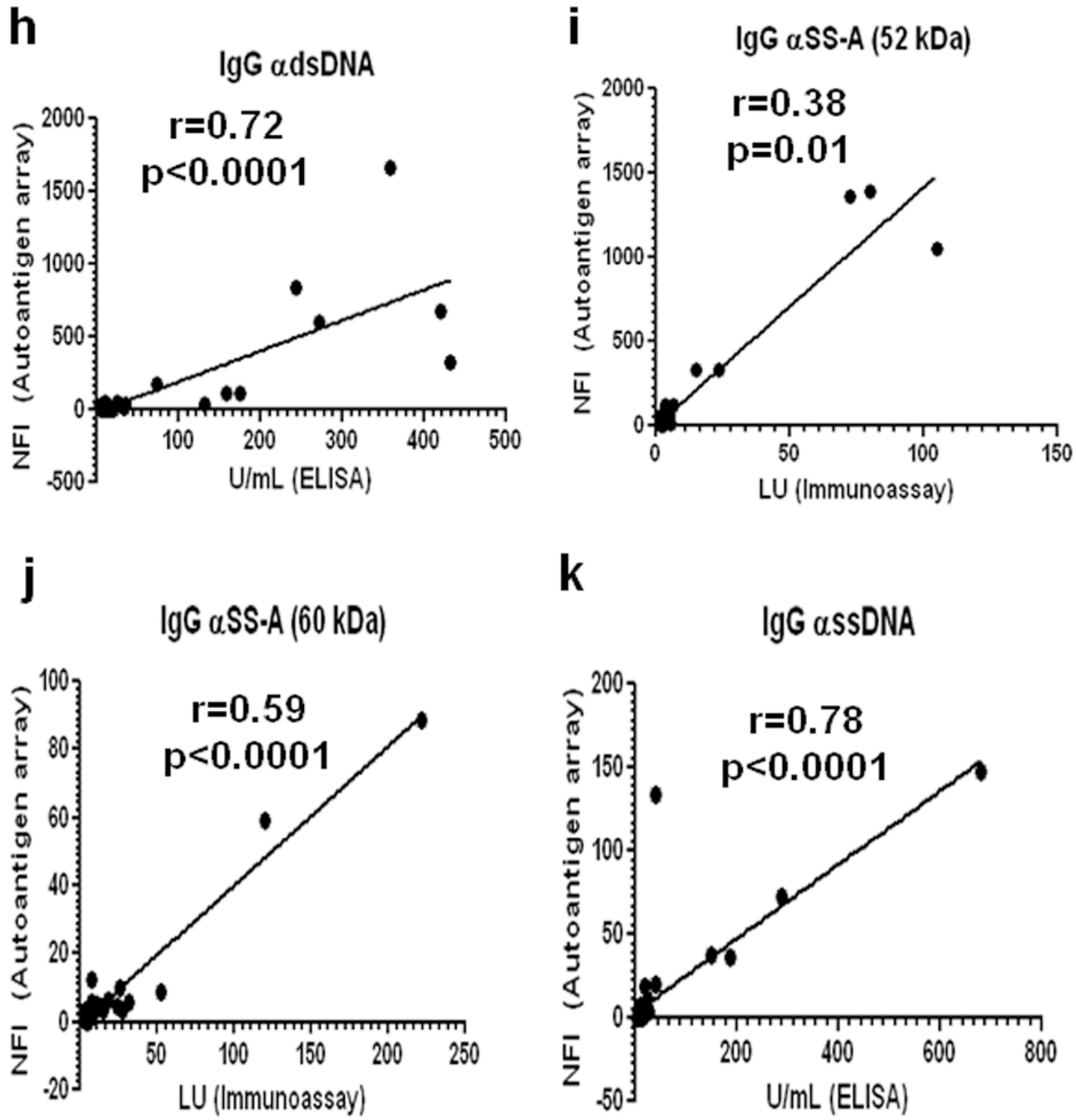
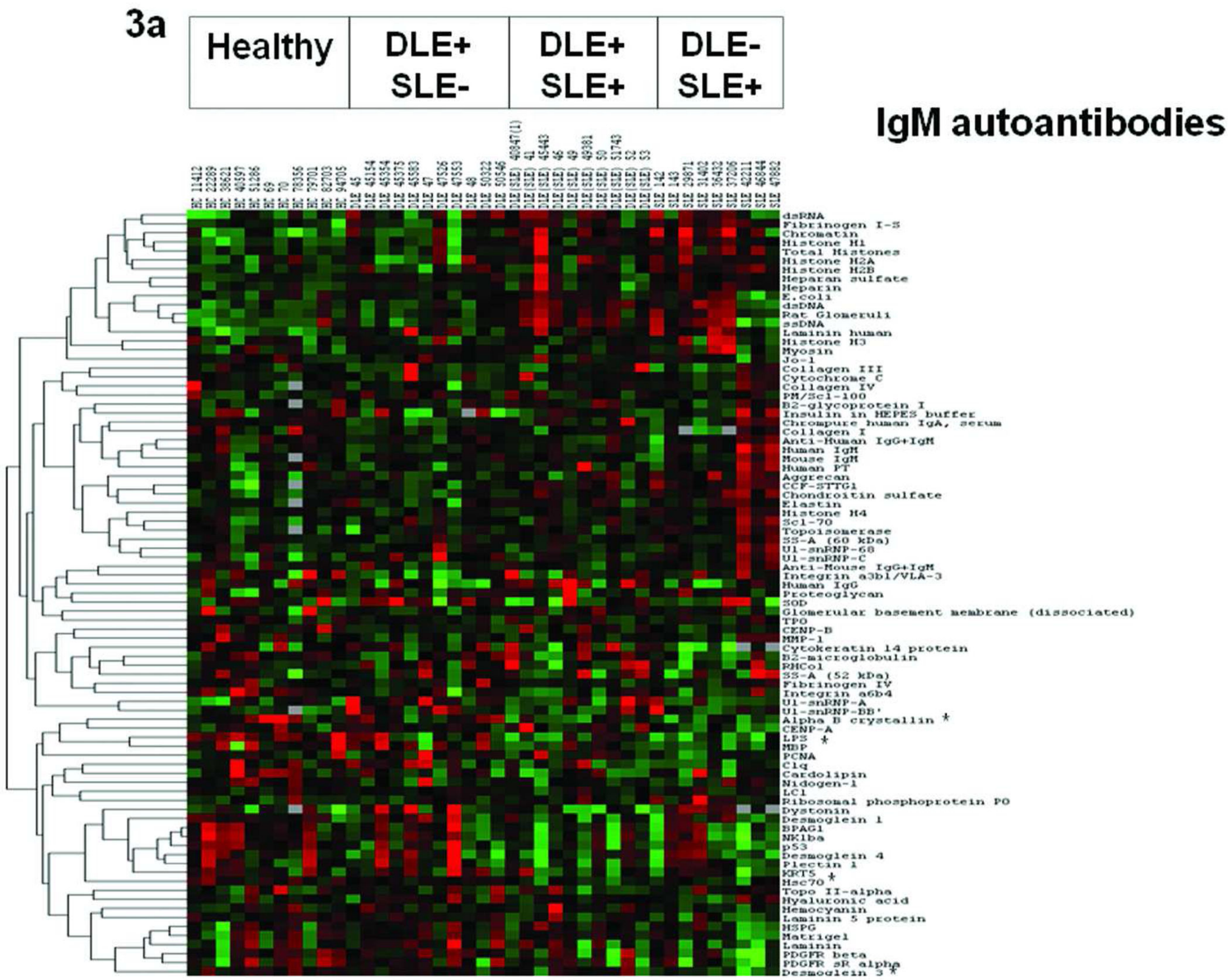


Figure 2. IgG autoantibodies against nuclear antigens in DLE and SLE sera using immunoassays
(**a–g**) We performed ELISAs and fluorescent immunoassays to measure IgG autoantibodies against dsDNA (**a**), ssDNA (**b**), SS-A (52 kDa) (**c**), SS-A (60 kDa) (**d**), U1-snRNP-BB’ (**e**), histones (**f**), and ANA (**g**). We performed one-way ANOVA with Tukey’s HSD test for multiple comparisons. (**h–k**) We generated correlation plots of immunoassay values and autoantigen array NFIs for each subject sample for anti-dsDNA (**h**), -SS-A (52 kDa) (**i**), -SS-A (60 kDa) (**j**), and -ssDNA (**k**). Spearman’s r and corresponding p-values were reported for each graph. *: p<0.05, **: p<0.005, ***: p<0.0005.


3a
Healthy
DLE+ SLE-
DLE+ SLE+
DLE- SLE+
IgM autoantibodies
dsRNA
Fibrinogen I-S
Chromatin
Histone H1
Total Histones
Histone H2A
Histone H2B
Heparan sulfate
Heparin
E.coli
dsDNA
Rat Glomeruli
ssDNA
Laminin human
Histone H3
Myosin
Jo-1
Collagen III
Cytochrome C
Collagen IV
PM/Scl-100
B2-glycoprotein I
Insulin in HEPES buffer
Chrompure human IgA, serum
Collagen I
Anti-Human IgG+IgM
Human IgM
Mouse IgM
Human PT
Aggrecan
CCF-STTG1
Chondroitin sulfate
Elastin
Histone H4
Scl-70
Topoisomerase
SS-A (60 kDa)
U1-snRNP-68
U1-snRNP-C
Anti-Mouse IgG+IgM
Integrin a3b1/VLA-3
Human IgG
Proteoglycan
SOD
Glomerular basement membrane (dissociated)
TPO
CENP-B
MMP-1
Cytokeratin 14 protein
B2-microglobulin
RNCol
SS-A (52 kDa)
Fibrinogen IV
Integrin a6b4
U1-snRNP-A
U1-snRNP-BB'
Alpha B crystallin *
CENP-A
LPS *
MBP
PCNA
C1q
Cardolipin
Nidogen-1
LC1
Ribosomal phosphoprotein P0
Dystonin
Desmoglein 1
BPAG1
NK1ba
p53
Desmoglein 4
Plectin 1
KRT5 *
Hsc70 *
Topo II-alpha
Hyaluronic acid
Hemocyanin
Laminin 5 protein
HSPG
Matrigel
Laminin
PDGFR beta
PDGFR sR alpha
Desmoglein 3 *

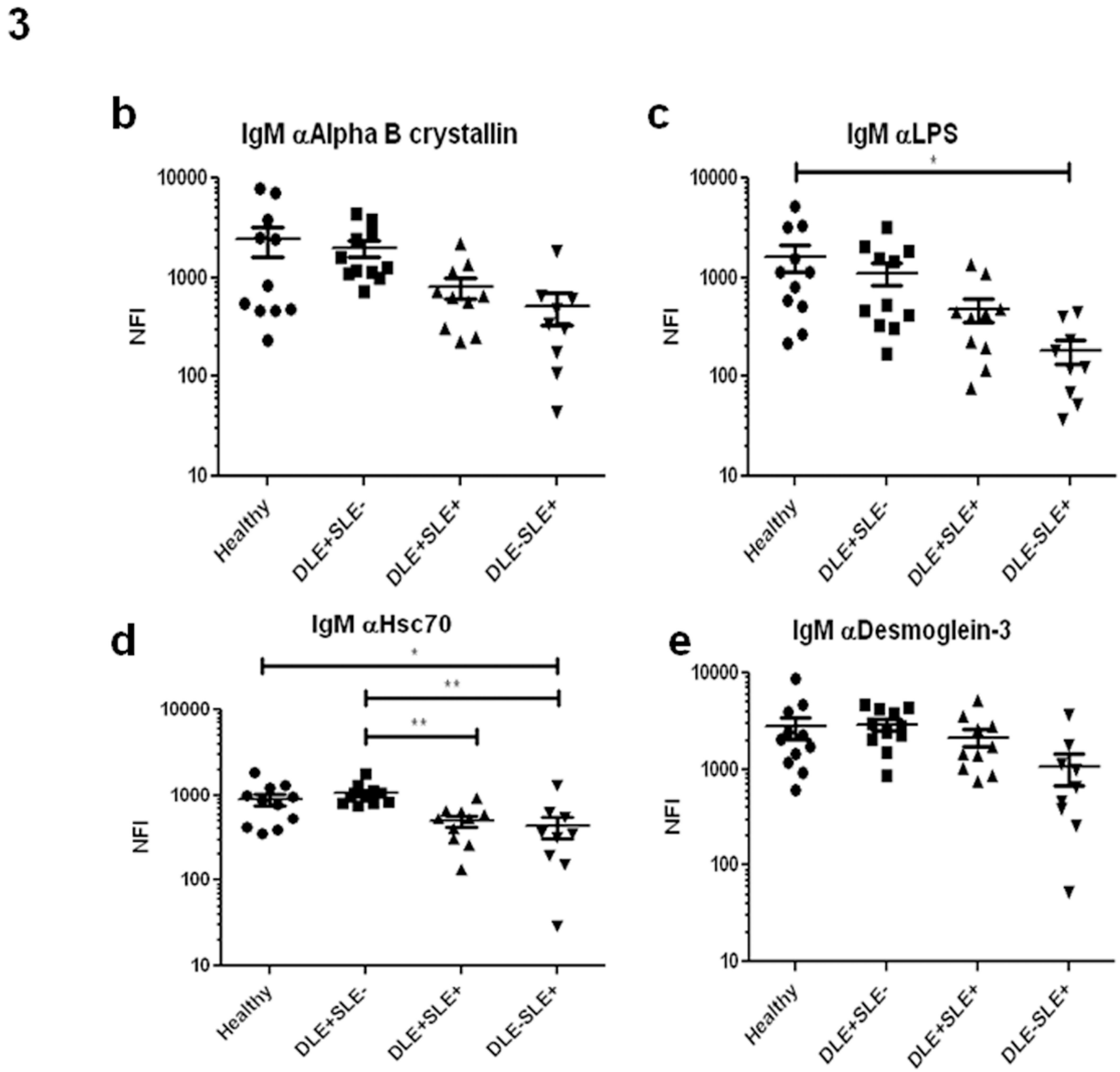


Figure 3. IgM autoantibody levels in sera of DLE and SLE subjects as determined by autoantigen arrays

(**a**) We generated a heat map summarizing IgM reactivities in the four groups. Green, black, and red represent NFIs below, close to, and above the mean, respectively. SAM analysis identified differentially expressed autoantibodies (*: q<0.05). (**b–e**) For each group, we plotted NFIs for IgM autoantibodies against alpha B crystallin (**b**), LPS (**c**), Hsc70 (**d**), and desmoglein-3 (**e**). We performed secondary analyses using one-way ANOVA with Tukey's HSD test for multiple comparisons. *: p<0.05, **: p<0.005.

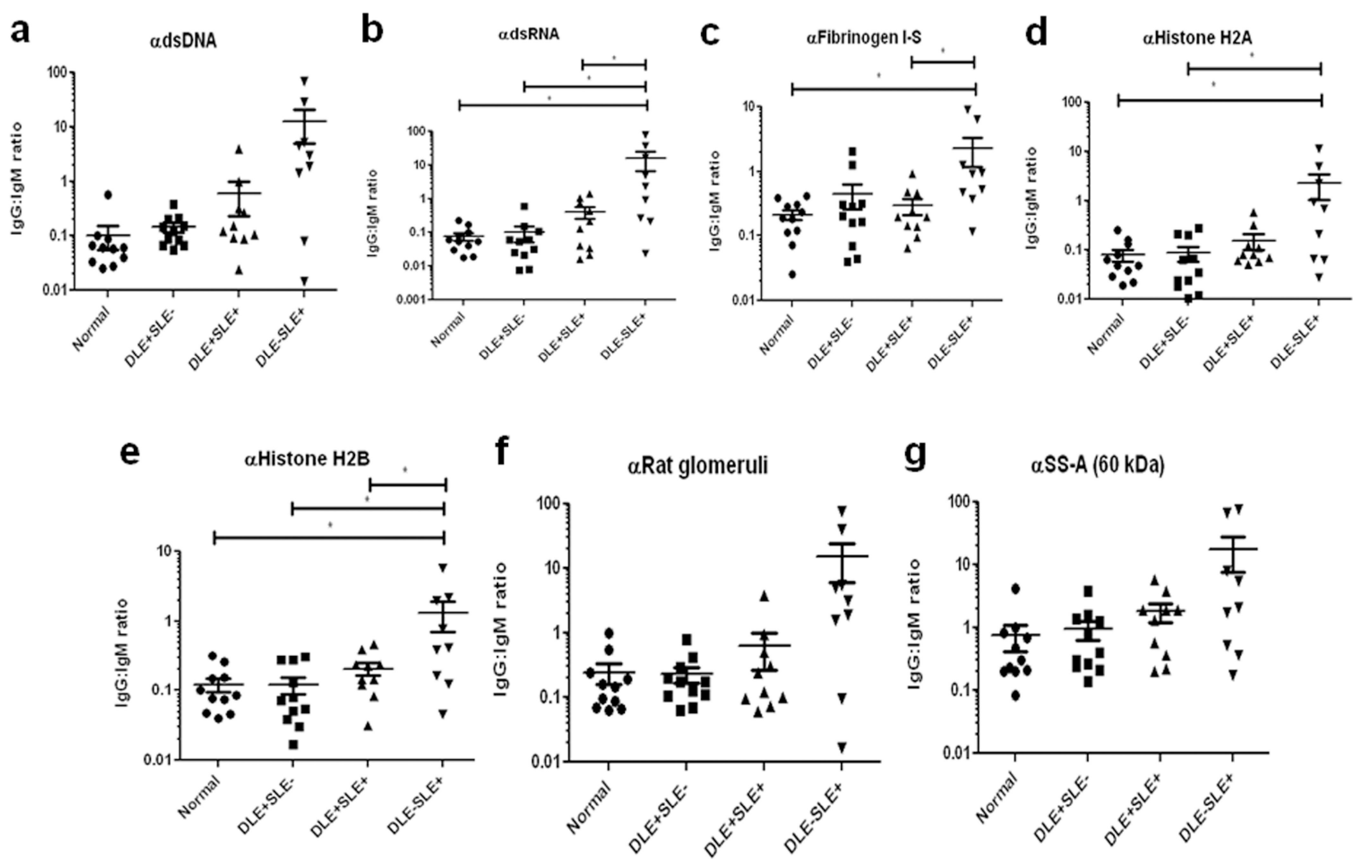


Figure 4. IgG:IgM ratios of autoantibodies against selected antigens as determined by autoantigen arrays

(**a–g**) For each group, we plotted ratios of IgG and IgM NFIs for autoantibodies against dsDNA (**a**), dsRNA (**b**), fibrinogen I-S (**c**), histone H2A (**d**), histone H2B (**e**), rat glomeruli (**f**), and SS-A (60 kDa) (**g**). We performed one-way ANOVA with Tukey's HSD test for multiple comparisons. *: $p<0.05$.

Table 1

Patient characteristics (Cohort used for autoantigen arrays).

	Normal	DLE+SLE−	DLE+SLE+	DLE-SLE+	p-value*
N	11	11	10	9	---
Gender (F)	10	11	9	8	0.78
Age at visit, mean (SD)	45 (11)	46 (12)	48 (12)	44 (11)	0.88
Ethnicity, N (%)					
Caucasian	5 (45)	4 (36)	4 (40)	1 (11)	0.42
African American	5 (45)	7 (64)	4 (40)	5 (56)	0.76
Hispanic	1 (10)	0 (0)	2 (20)	3 (33)	0.15
CLASI activity score, mean (SD)**	N/A	9 (9)	10 (7)	N/A	0.86
CLASI damage score, mean (SD)**	N/A	7 (5)	10 (9)	N/A	0.35
SLEDAI score, mean (SD)***	N/A	3 (3)	4 (2)	2 (4)†	0.38
Lupus medications at study visit, N (%)***					
Topical/intralesional corticosteroids	N/A	5 (45)	7 (70)	0 (0)	0.005
Hydroxychloroquine	N/A	6 (54)	6 (60)	3 (33)	0.54
Chloroquine	N/A	3 (27)	1 (10)	0 (0)	0.35
Quinacrine	N/A	4 (36)	2 (20)	0 (0)	0.19
Methotrexate	N/A	2 (18)	0 (0)	1 (11)	0.62
Prednisone	N/A	0 (0)	4 (40)	6 (67)	0.003
Mycophenolate mofetil	N/A	1 (9)	5 (50)	1 (11)	0.09
Efalizumab	N/A	0 (0)	1 (10)	0 (0)	0.63
Leflunomide	N/A	0 (0)	1 (10)	0 (0)	0.63
None	N/A	2 (18)	0 (0)	2 (22)	0.42
SLE criteria, N (%)***					
Malar rash	N/A	0 (0)	2 (20)	0 (0)	0.19
Discoid rash	N/A	11 (100)	10 (100)	0 (0)	<0.00001
Photosensitivity	N/A	7 (64)	9 (90)	0 (0)	0.0001
Oral ulcers	N/A	2 (18)	6 (60)	0 (0)	0.008
Arthritis	N/A	2 (18)	6 (60)	6 (67)	0.07

	Normal	DLE+SLE−	DLE+SLE+	DLE-SLE+	p-value*
Serositis	N/A	0 (0)	3 (30)	4 (44)	0.04
Renal disorder	N/A	0 (0)	4 (40)	6 (67)	0.01
Neurological disorder	N/A	0 (0)	0 (0)	1 (11)	0.30
Hematological disorder	N/A	1 (9)	8 (80)	9 (100)	0.00002
ANA	N/A	4 (36)	10 (100)	9 (100)	0.00008
Immunological disorder	N/A	0 (0)	7 (70)	9 (100)	<0.00001

*p-values were calculated using either one-way ANOVA tests (continuous variables) or Fisher's exact tests (categorical variables).

**p-values were calculated using Student's t-tests between DLE+SLE− and DLE+SLE+ groups since they were the only groups whose skin lesions were evaluated with CLASI.

***p-value was calculated using one-way ANOVA tests among DLE+SLE−, DLE+SLE+, and DLE-SLE+ groups since they were the only groups whose disease activities were assessed using SLEDAI, and whose lupus medications and SLE criteria were recorded.

†SLEDAI values were not calculated for five DLE-SLE+ subjects.

Abbreviations: ANA = anti-nuclear antibody; CLASI = Cutaneous Lupus Disease Activity and Severity Index; DLE = discoid lupus erythematosus; SLE = systemic lupus erythematosus; SLEDAI = Systemic Lupus Erythematosus Disease and Activity Index

Table 2

Patient characteristics (Cohort used for validation).

	Normal	DLE+SLE−	DLE+SLE+	DLE-SLE+§	p-value*
N	22	23	17	18	---
Gender (F)	19	20	16	16	0.93
Age at visit, mean (SD)	43 (11)	43 (11)	44 (12)	40 (13)	0.76
Ethnicity, N (%)					
Caucasian	6 (27)	6 (26)	4 (23)	2 (11)	0.61
African American	14 (64)	16 (70)	10 (59)	11 (61)	0.90**
Hispanic	2 (9)	1 (4)	3 (18)	5 (28)	0.15
CLASI activity score, mean (SD)***	N/A	7 (7)	10 (8)†	N/A	0.27
CLASI damage score, mean (SD)***	N/A	8 (5)	11 (8)†	N/A	0.13
SLEDAI score, mean (SD)****	N/A	1 (2)	4 (2)	2 (3)‡	0.02
Lupus medications at study visit, N (%)****					
Topical/intralesional corticosteroids	N/A	9 (39)	9 (53)	1 (6)	0.005
Hydroxychloroquine	N/A	14 (61)	11 (65)	6 (33)	0.12**
Chloroquine	N/A	3 (13)	1 (6)	0 (0)	0.30
Quinacrine	N/A	4 (17)	2 (12)	0 (0)	0.23
Methotrexate	N/A	2 (9)	0 (0)	1 (6)	0.77
Prednisone	N/A	0 (0)	7 (41)	12 (67)	<0.00001
Mycophenolate mofetil	N/A	1 (4)	7 (41)	3 (17)	0.02
Efalizumab	N/A	0 (0)	1 (6)	0 (0)	0.30
Leflunomide	N/A	0 (0)	1 (6)	0 (0)	0.30
Cyclophosphamide	N/A	0 (0)	0 (0)	1 (6)	0.61
None	N/A	6 (26)	0 (0)	3 (17)	0.09
SLE criteria, N (%)***					
Malar rash	N/A	1 (4)	4 (24)	1 (6)	0.14
Discoid rash	N/A	23 (100)	17 (100)	0 (0)	<0.00001
Photosensitivity	N/A	15 (65)	15 (88)	2 (11)	<0.00001
Oral ulcers	N/A	3 (13)	10 (59)	2 (11)	0.002

	Normal	DLE+SLE−	DLE+SLE+	DLE-SLE+[§]	p-value[*]
Arthritis	N/A	2 (9)	9 (53)	10 (56)	0.001
Serositis	N/A	0 (0)	4 (24)	7 (39)	0.001
Renal disorder	N/A	0 (0)	5 (29)	13 (72)	<0.00001
Neurological disorder	N/A	0 (0)	0 (0)	1 (6)	0.60
Hematological disorder	N/A	3 (13)	13 (76)	15 (83)	<0.00001
ANA	N/A	8 (35)	16 (94)	18 (100)	<0.00001[**]
Immunological disorder	N/A	0 (0)	14 (82)	18 (100)	<0.00001

§ 1 DLE-SLE+ subject met three criteria (renal (kidney biopsy consistent with lupus nephritis), ANA, immunological disorder).

* p-values were calculated using either one-way ANOVA tests (continuous variables) or Fisher's exact test (categorical variables).

** p-values were calculated using X^2-tests.

*** p-values were calculated using Student's t-tests between DLE+SLE− and DLE+SLE+ groups since they were the only groups whose skin lesions were evaluated with CLASI.

**** p-value was calculated using one-way ANOVA tests among DLE+SLE−, DLE+SLE+, and DLE-SLE+ groups since they were the only groups whose disease activities were assessed using SLEDAI, and whose lupus medications and SLE criteria were recorded.

† CLASI activity and damage scores were not calculated for one DLE+SLE+ subject.

‡ SLEDAI values were not calculated for seven DLE-SLE+ subjects.

Abbreviations: ANA = anti-nuclear antibody; CLASI = Cutaneous Lupus Disease Activity and Severity Index; DLE = discoid lupus erythematosus; SLE = systemic lupus erythematosus; SLEDAI = Systemic Lupus Erythematosus Disease and Activity Index