IgG and IgM Autoantibody Differences in Discoid and Systemic Lupus Patients

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Systemic lupus erythematosus (SLE) patients with discoid lupus erythematosus (DLE) were reported to have milder disease. To test this observation, we used 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 (10 against nuclear antigens) and 4 IgM autoantibodies that were differentially expressed (q-value<0.05). DLE−SLE+ subjects had higher IgG autoantibodies against double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), histone H2A and H2B, and SS-A (52 kDa) compared with 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 compared with 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 nonpathogenic.

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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; Prystowsky and Gilliam, 1975; Merola *et al.*, 2011). In all, 21–63% of DLE patients have antinuclear antibodies (ANAs) (Millard and Rowell, 1979; Callen, 1982; Wallace, 1993), but <5% have significant ANA titers (>1:320) seen in SLE patients (Costner and Sontheimer, 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. To provide comprehensive autoantibody profiles of patients, Robinson *et al.* (2002) 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- to 8-fold greater sensitivity in detecting the presence of autoantibodies than ELISAs.

Autoantigen arrays have generated autoantibody profiles that can discriminate patient groups and provide 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).

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Abbreviations: ACR, American College of Rheumatology; ANA, antinuclear antibody; DLE, discoid lupus erythematosus; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; HSPG, heparan sulfate proteoglycan; SAM, significance analysis of microarray; SLE, systemic lupus erythematosus; ssDNA, single-stranded DNA

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Comparing autoantibody profiles in DLE and SLE subjects may uncover autoantibodies that distinguish these two entities, and shed light on 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 (SAMs) 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, desmoglein-3, dsDNA, double-stranded RNA (dsRNA), fibrinogen I-S, histone H1, histone H2A, histone H2B, platelet-derived growth factor receptor beta, rat glomeruli, SS-A (52 kDa), SS-A (60 kDa), ssDNA, and U1-snRNP-BB′. Ten of these autoantibodies targeted nuclear antigens (anti-centromere protein-A, -dsDNA, -dsRNA, -histone H1, -histone H2A, -histone H2B, -SS-A (52 kDa), -SS-A (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-centromere protein-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. It is noteworthy that anti-dsDNA, -dsRNA, -histone H2A, -histone H2B, -SS-A (52 kDa), and -ssDNA antibodies were significantly higher in DLE−SLE+ subjects compared

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)**	NA	9 (9)	10 (7)	NA	0.86
CLASI damage score, mean (SD)**	NA	7 (5)	10 (9)	NA	0.35
SLEDAI score, mean (SD)***	NA	3 (3)	4 (2)	2 (4)[1]	0.38
Lupus medications at study visit, N *(%)****					
Topical/intralesional corticosteroids	NA	5 (45)	7 (70)	0 (0)	0.005
Hydroxychloroquine	NA	6 (54)	6 (60)	3 (33)	0.54
Chloroquine	NA	3 (27)	1 (10)	0 (0)	0.35
Quinacrine	NA	4 (36)	2 (20)	0 (0)	0.19
Methotrexate	NA	2 (18)	0 (0)	1 (11)	0.62
Prednisone	NA	0 (0)	4 (40)	6 (67)	0.003
Mycophenolate mofetil	NA	1 (9)	5 (50)	1 (11)	0.09
Efalizumab	NA	0 (0)	1 (10)	0 (0)	0.63
Leflunomide	NA	0 (0)	1 (10)	0 (0)	0.63
None	NA	2 (18)	0 (0)	2 (22)	0.42
SLE criteria, N *(%)****					
Malar rash	NA	0 (0)	2 (20)	0 (0)	0.19
Discoid rash	NA	11 (100)	10 (100)	0 (0)	<0.00001
Photosensitivity	NA	7 (64)	9 (90)	0 (0)	0.0001
Oral ulcers	NA	2 (18)	6 (60)	0 (0)	0.008
Arthritis	NA	2 (18)	6 (60)	6 (67)	0.07
Serositis	NA	0 (0)	3 (30)	4 (44)	0.04
Renal disorder	NA	0 (0)	4 (40)	6 (67)	0.01
Neurological disorder	NA	0 (0)	0 (0)	1 (11)	0.30
Hematological disorder	NA	1 (9)	8 (80)	9 (100)	0.00002
ANA	NA	4 (36)	10 (100)	9 (100)	0.00008
Immunological disorder	NA	0 (0)	7 (70)	9 (100)	<0.00001

Abbreviations: ANA, antinuclear antibody; ANOVA, analysis of variance; CLASI, Cutaneous Lupus Disease Activity and Severity Index; DLE, discoid lupus erythematosus; NA, not applicable; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease and Activity Index.
[1]SLEDAI values were not calculated for five DLE−SLE+ subjects.
**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, because 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, because they were the only groups whose disease activities were assessed using SLEDAI, and whose lupus medications and SLE criteria were recorded.

Table 2. Patient characteristics (cohort used for validation)

	Normal	DLE+SLE−	DLE+SLE+	DLE−SLE+[1]	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)***	NA	7 (7)	10 (8)[2]	NA	0.27
CLASI damage score, mean (SD)***	NA	8 (5)	11 (8)[2]	NA	0.13
SLEDAI score, mean (SD)****	NA	1 (2)	4 (2)	2 (3)[3]	0.02
*Lupus medications at study visit, N (%)**** *					
Topical/intralesional corticosteroids	NA	9 (39)	9 (53)	1 (6)	0.005
Hydroxychloroquine	NA	14 (61)	11 (65)	6 (33)	0.12**
Chloroquine	NA	3 (13)	1 (6)	0 (0)	0.30
Quinacrine	NA	4 (17)	2 (12)	0 (0)	0.23
Methotrexate	NA	2 (9)	0 (0)	1 (6)	0.77
Prednisone	NA	0 (0)	7 (41)	12 (67)	<0.00001
Mycophenolate mofetil	NA	1 (4)	7 (41)	3 (17)	0.02
Efalizumab	NA	0 (0)	1 (6)	0 (0)	0.30
Leflunomide	NA	0 (0)	1 (6)	0 (0)	0.30
Cyclophosphamide	NA	0 (0)	0 (0)	1 (6)	0.61
None	NA	6 (26)	0 (0)	3 (17)	0.09
*SLE criteria, N (%)****					
Malar rash	NA	1 (4)	4 (24)	1 (6)	0.14
Discoid rash	NA	23 (100)	17 (100)	0 (0)	<0.00001
Photosensitivity	NA	15 (65)	15 (88)	2 (11)	<0.00001
Oral ulcers	NA	3 (13)	10 (59)	2 (11)	0.002
Arthritis	NA	2 (9)	9 (53)	10 (56)	0.001
Serositis	NA	0 (0)	4 (24)	7 (39)	0.001
Renal disorder	NA	0 (0)	5 (29)	13 (72)	<0.00001
Neurological disorder	NA	0 (0)	0 (0)	1 (6)	0.60
Hematological disorder	NA	3 (13)	13 (76)	15 (83)	<0.00001
ANA	NA	8 (35)	16 (94)	18 (100)	<0.00001**
Immunological disorder	NA	0 (0)	14 (82)	18 (100)	<0.00001

Abbreviations: ANA, antinuclear antibody; ANOVA, analysis of variance; CLASI, Cutaneous Lupus Disease Activity and Severity Index; DLE, discoid lupus erythematosus; NA, not applicable; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease and Activity Index.
[1]1 DLE-SLE+ subject met three criteria (renal (kidney biopsy consistent with lupus nephritis), ANA, immunological disorder).
[2]CLASI activity and damage scores were not calculated for one DLE+SLE+ subject.
[3]SLEDAI values were not calculated for seven DLE−SLE+ subjects.
**P*-values were calculated using either one-way ANOVA tests (continuous variables) or Fisher's exact test (categorical variables).
***P*-values were calculated using χ^2 tests.
****P*-values were calculated using Student's *t*-tests between DLE+SLE− and DLE+SLE+ groups, because 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, because they were the only groups whose disease activities were assessed using SLEDAI, and whose lupus medications and SLE criteria were recorded.

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 ($P<0.05$) (Supplementary Figure S1a–c online). 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 (Supplementary Figure S1d online).

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, -platelet-derived growth factor receptor sR alpha, -SS-A (52 kDa), and -U1-snRNP-A) that were significantly upregulated in DLE+SLE− subjects ($q<0.05$) (Supplementary Figure S2a–j online). 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 compared with 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 upregulated 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 downregulated 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, and -lipopolysaccharide) that were differentially expressed in the four groups (asterisked in Figure 3a). IgM autoantibodies against alpha B crystallin and lipopolysaccharide were highest in healthy controls, followed by DLE+SLE−, DLE+SLE+, and DLE−SLE+ subjects (Figure 3b and c). IgM autoantibodies against heat-shock cognate 70 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 upregulated IgM autoantibodies in DLE+SLE− subjects compared with 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 compared with 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+ versus 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 UV 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 (Gilliam *et al.*, 1974; Prystowsky and Gilliam, 1975; Callen, 1982). A comparison of our DLE−SLE+ and DLE+SLE+ subjects reveals that a smaller portion of DLE+SLE+ subjects had renal and neurological disease, as well as 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).

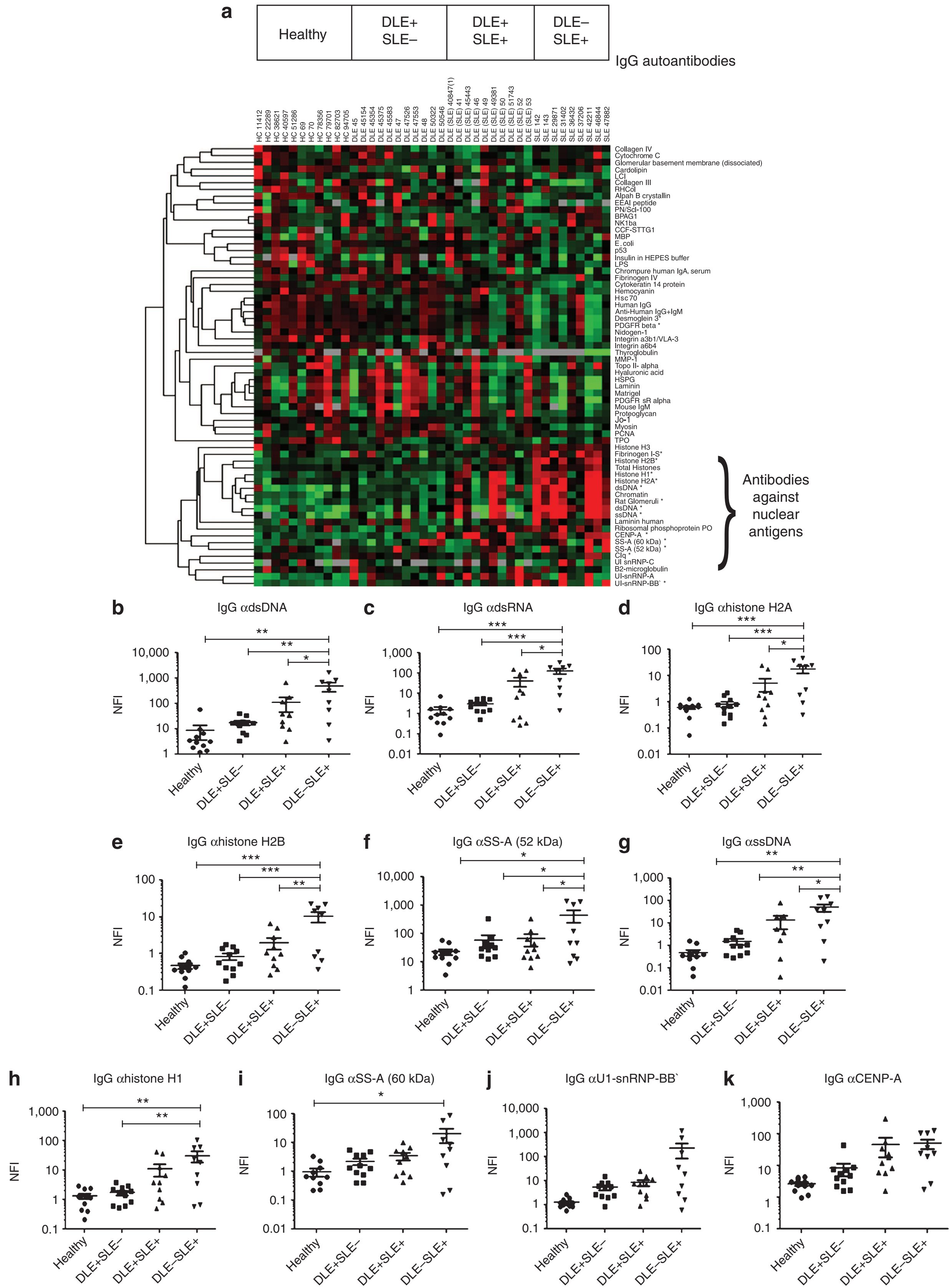
Our data showing elevated IgG autoantibodies against nuclear antigens in SLE subjects are consistent with previous observations in SLE patients (Adu *et al.*, 1981). The DLE−SLE+ group, which had the highest percentage of renal disease, expressed the highest autoantibodies against dsDNA and histone proteins, 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 (Provost *et al.*, 1985; Wallace, 1993; Lee *et al.*, 1994) 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 10 upregulated 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. On the basis of 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 compared with 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. In addition, 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 whether 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,

Figure 1. IgG autoantibody levels in discoid lupus erythematosus (DLE) and systemic lupus erythematosus (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 net fluorescence intensities (NFIs) below, close to, and above the mean, respectively. Significance analysis of microarray (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 double-stranded DNA (dsDNA) (**b**), double-stranded RNA (dsRNA) (**c**), histone H2A (**d**), histone H2B (**e**), SS-A (52 kDa) (**f**), single-stranded DNA (ssDNA) (**g**), histone H1 (**h**), SS-A (60 kDa) (**i**), U1-snRNP-BB′ (**j**), and centromere protein-A (CENP-A) (**k**). We performed secondary analyses using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test for multiple comparisons. *$P<0.05$, **$P<0.005$, ***$P<0.0005$.


a
Healthy
DLE+ SLE−
DLE+ SLE+
DLE− SLE+
IgG autoantibodies
Antibodies against nuclear antigens
b
IgG αdsDNA
c
IgG αdsRNA
d
IgG αhistone H2A
e
IgG αhistone H2B
f
IgG αSS-A (52 kDa)
g
IgG αssDNA
h
IgG αhistone H1
i
IgG αSS-A (60 kDa)
j
IgG αU1-snRNP-BB`
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IgG αCENP-A
NFI
Healthy
DLE+SLE−
DLE+SLE+
DLE−SLE+

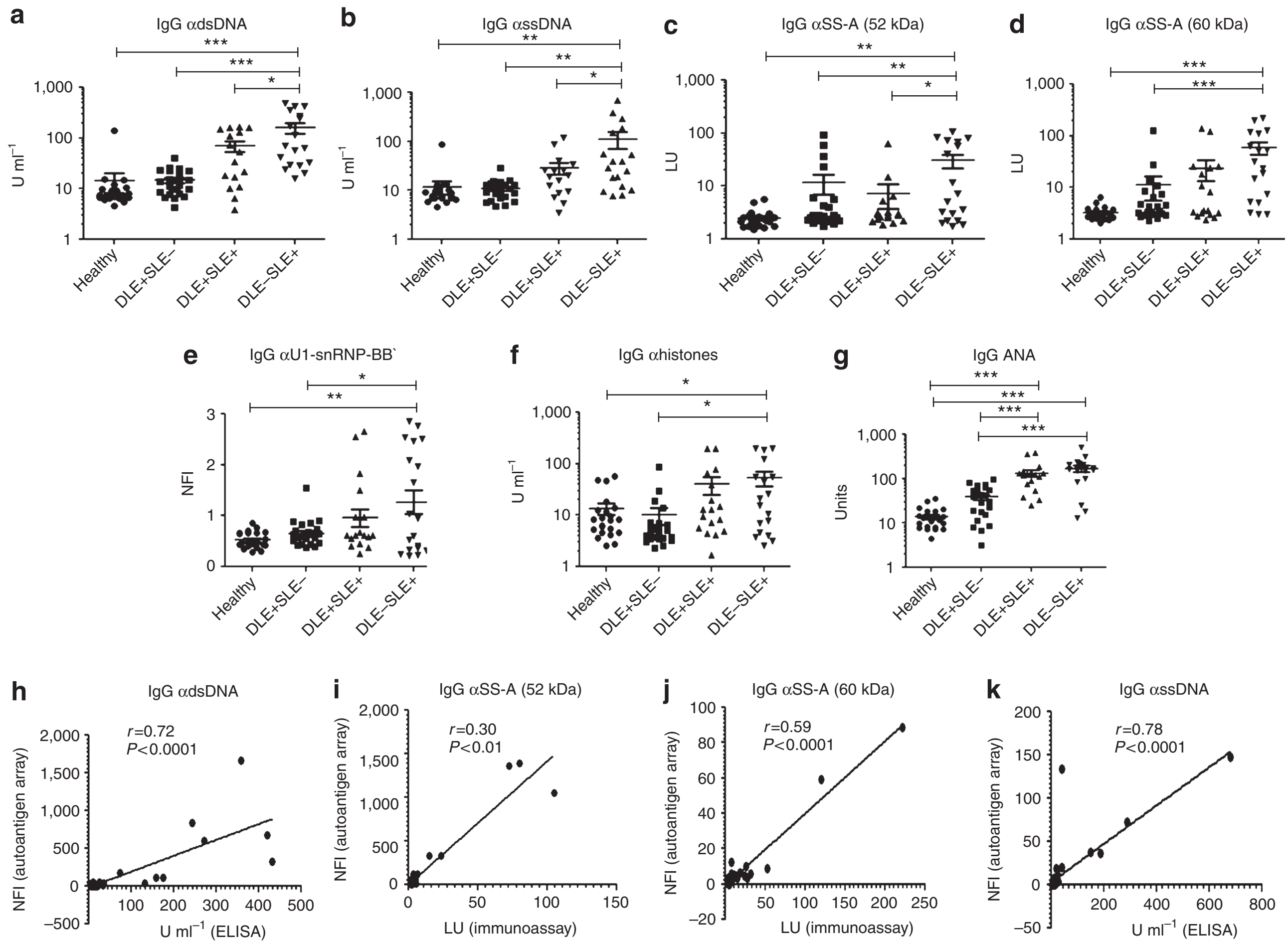

Figure 2. IgG autoantibodies against nuclear antigens in discoid lupus erythematosus (DLE) and systemic lupus erythematosus (SLE) sera using immunoassays. (**a–g**) We performed ELISAs and fluorescent immunoassays to measure IgG autoantibodies against double-stranded DNA (dsDNA) (**a**), single-stranded DNA (ssDNA) (**b**), SS-A (52 kDa) (**c**), SS-A (60 kDa) (**d**), U1-snRNP-BB′ (**e**), histones (**f**), and antinuclear antibody (ANA) (**g**). We performed one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test for multiple comparisons. (**h–k**) We generated correlation plots of immunoassay values (U ml^{-1} or luminous unit (LU)) and autoantigen array net fluorescence intensities (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 are reported for each graph. *$P<0.05$, **$P<0.005$, ***$P<0.0005$.

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 (Prystowsky and Gilliam, 1975; Merola *et al.*, 2011). 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. Although 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 multicenter 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. Although 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

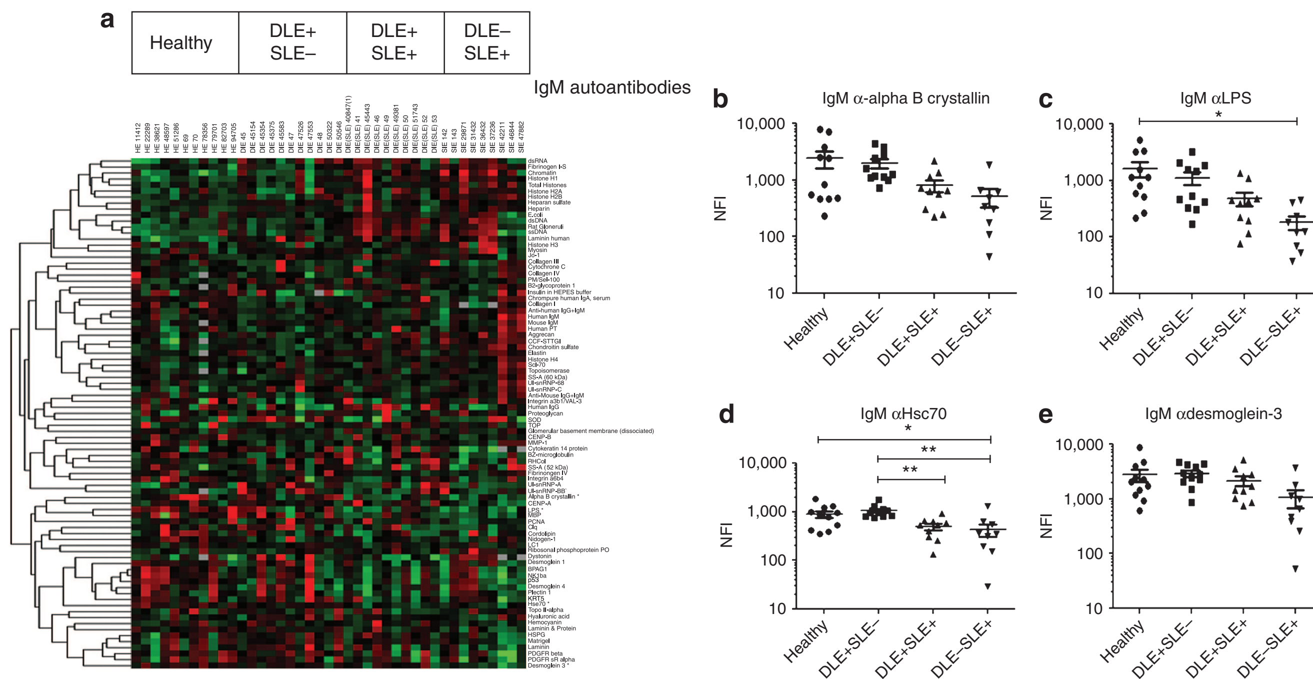

Figure 3. IgM autoantibody levels in sera of discoid lupus erythematosus (DLE) and systemic lupus erythematosus (SLE) subjects as determined by autoantigen arrays. (**a**) We generated a heat map summarizing IgM reactivities in the four groups. Green, black, and red represent net fluorescence intensities (NFIs) below, close to, and above the mean, respectively. Significance analysis of microarray (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**), lipopolysaccharide (LPS) (**c**), heat-shock cognate 70 (Hsc70) (**d**), and desmoglein-3 (**e**). We performed secondary analyses using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test for multiple comparisons. *$P<0.05$, **$P<0.005$.

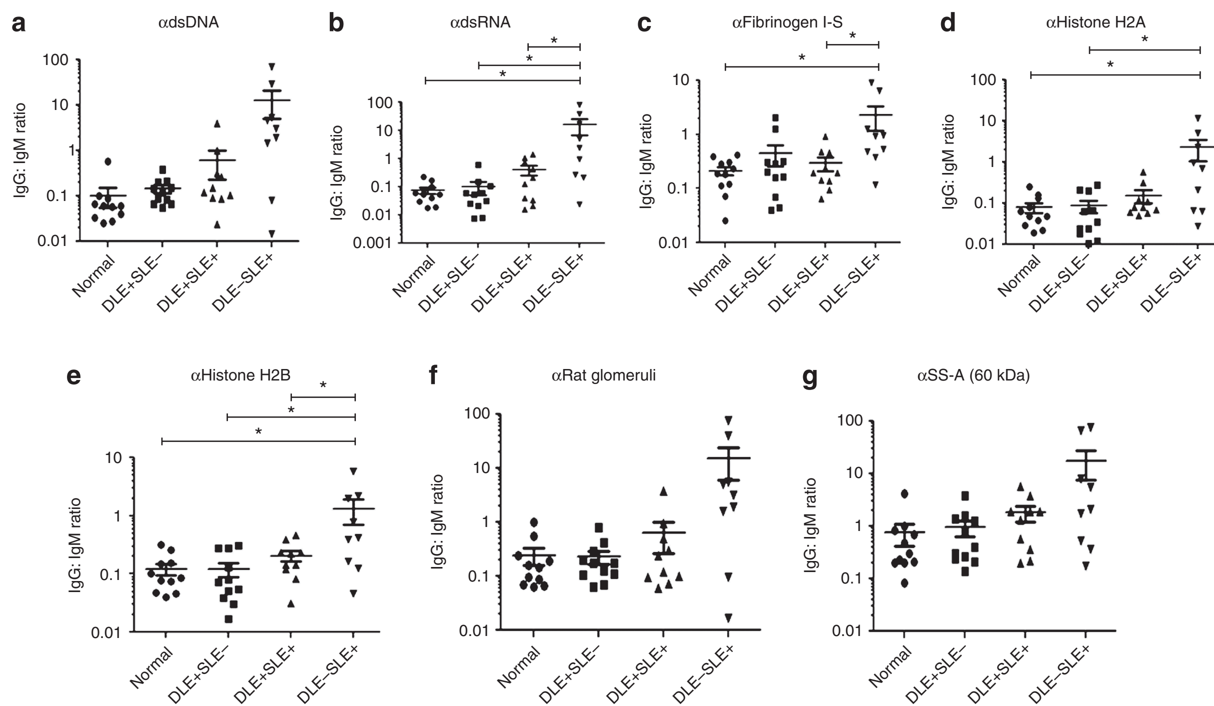

Figure 4. IgG:IgM ratios of autoantibodies against selected antigens as determined by autoantigen arrays. (**a-g**) For each group, we plotted ratios of IgG and IgM net fluorescence intensities (NFIs) for autoantibodies against double-stranded DNA (dsDNA) (**a**), double-stranded RNA (dsRNA) (**b**), fibrinogen I-S (**c**), histone H2A (**d**), histone H2B (**e**), rat glomeruli (**f**), and SS-A (60 kDa) (**g**). We performed one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test for multiple comparisons. *$P<0.05$.

arrays and immunoassays. The downregulation 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, heat-shock cognate 70, and lipopolysaccharide were noted in both SLE groups. DLE−SLE+ subjects had the highest IgG:IgM ratios against autoantibodies against mostly nuclear antigens. We have hypothesized nonpathogenic 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. A total of 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. Although 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 were collected after centrifugation of blood samples at 3,000 r.p.m. 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 (Supplementary Table S1 online), 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, 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 by those from anti-human IgG in the array and multiplying the ratio by 1,000. We excluded IgG and IgM autoantibodies whose levels were <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, 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 lipopolysaccharide (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).

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 the Cluster and Treeview software (http://rana.lbl.gov/EisenSoftware.htm). Subject characteristics were compared using one-way analysis of variance 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 analysis of variance with Tukey's honestly significant difference 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 (analysis of variance, 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), <0.05.

CONFLICTS OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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