Defective B cell tolerance checkpoints in systemic lupus erythematosus

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A cardinal feature of systemic lupus erythematosus (SLE) is the development of autoantibodies. The first autoantibodies described in patients with SLE were those specific for nuclei and DNA, but subsequent work has shown that individuals with this disease produce a panoply of different autoantibodies. Thus, one of the constant features of SLE is a profound breakdown in tolerance in the antibody system. The appearance of self–reactive antibodies in SLE precedes clinical disease, but where in the B cell pathway tolerance is first broken has not been defined. In healthy humans, autoantibodies are removed from the B cell repertoire in two discrete early checkpoints in B cell development. We found these checkpoints to be defective in three adolescent patients with SLE. 25–50% of the mature naive B cells in SLE patients produce self–reactive antibodies even before they participate in immune responses as compared with 5–20% in controls. We conclude that SLE is associated with abnormal early B cell tolerance.

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Abbreviations used: aa, amino acids; ANA, antinuclear antibody; IFA, indirect immunofluorescence assay; IgH, Ig heavy; IgL, Ig light; SLE, systemic lupus erythematosus; PS, phosphatidylserine.

Systemic lupus erythematosus (SLE) is a multigene autoimmune disorder characterized by a constellation of clinical abnormalities (1–3) that can affect nearly all of the components of the immune system (4–8). However, a hallmark of SLE is the production of autoantibodies that are involved in pathogenic immune complex formation and deposit in the kidneys of patients (9). The first autoantibodies described in patients with SLE were those specific for nuclei and DNA (10), but subsequent work has shown that individuals with this disease produce a diverse group of autoantibodies (9, 11), predominantly somatically mutated, class-switched IgGs. These antibodies are produced by antigen-experienced B cells, implicating abnormalities in tolerance in late stage B cells, but it has not been determined where B cell tolerance is first broken in SLE (12).

In healthy individuals, B cells that express autoreactive antibodies including antinuclear antibodies (ANAs) and polyreactive antibodies represent 55–75% of the highly diverse repertoire that is constantly generated by random Ig gene rearrangement during early B cell development in the BM. To ensure self–tolerance, autoreactive B cells producing such potentially harmful antibodies are efficiently removed from the naive repertoire at two checkpoints (13). The first checkpoint is at the immature B cell stage in the BM where the majority of polyreactive and ANAs are lost. The second checkpoint is in the periphery before maturation of new emigrant B cells into naive immunocompetent lymphocytes. Three mechanisms have been described to silence developing autoreactive B cells at the first checkpoint for self–tolerance in the bone marrow: deletion, anergy, and receptor editing (14–17). How tolerance is established at the second checkpoint in the periphery is less clear, and both positive and negative selection mechanisms have been proposed (18–23). Although the mechanisms that silence autoreactive B cells at the two checkpoints might be very different, failure to remove autoantibodies at either stage would likely result in increased numbers of naive circulating self-reactive B cells in the periphery and, therefore, potentially increased susceptibility to autoimmunity.

Here, we show that SLE is associated with a failure to establish self-tolerance during early B cell development leading to increased num-

S. Yurasov and H. Wardemann contributed equally to this work. The online version of this article contains supplemental material.

Table I. Patient characteristics

	SLE100	SLE101	SLE122
Gender	F	M	F
Age at diagnosis (yr)	15	15	9
Clinical features	arthritis	myocarditis	chorea
Lupus nephritis	IIB	III	IIB
Hematology	L, A, T	A	L, A
Serology	D, P, Sm, RNP	P	D
ANA	+	+	+
C3/C4	low	low	low
Treatment	none	none	none
Family history	maternal SLE	maternal IDDM, paternal MS	none

A, anemia; D, anti-dsDNA antibody; L, leukopenia; P, antiphospholipid antibody; RNP, anti-RNP antibody; Sm, anti-Smith antibody; T, thrombocytopenia.

bers of autoreactive mature naive B cells circulating in peripheral blood of untreated patients.

RESULTS

Antibody cloning from single purified new emigrant and mature naive B cells of patients with SLE

To examine early B cell tolerance checkpoints in patients with SLE, we cloned, expressed, and tested the reactivity of 222 antibodies from single B cells derived from peripheral blood of three untreated adolescent SLE patients (Supplemental Materials and methods, available at http://www.jem.org/cgi/content/full/jem.20042251/DC1). All three SLE patients were diagnosed based on the Revised Criteria of the American College of Rheumatology and their clinical diagnostic features reflected the heterogeneity of this systemic autoimmune disease (Table I, reference 24, and Supplemental Materials and methods).

Antibodies were cloned from cDNA libraries created from single cells purified from newly emigrated (CD19$^+$ CD10$^+$IgM$^+$CD27$^-$) and mature naive B cell (CD19$^+$ CD10$^-$IgM$^+$CD27$^-$) compartments (references 13, 25–27 and Tables S1–S6 and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20042251/DC1). The sorted cells lacked CD27, a memory B cell marker, and the cloned antibodies were not somatically mutated or clonally related as determined by sequence analysis (references 26, 27 and Tables S1–S6). Therefore, these B cells were naive and showed no evidence of having been highly clonally expanded during their development.

Ig gene usage abnormalities are not consistent among patients with SLE

Abnormalities in Ig gene usage have been observed in SLE patients, and there is no evidence for changes in the repertoire between adolescents and adults (28–38). Therefore, we compared the Ig gene repertoire and Ig heavy (IgH) chain characteristics such as CDR3 length and positive charges, which have been associated with autoreactivity, between new emigrant and mature naive B cells from SLE patients

and our previously published healthy controls (Fig. 1 and reference 13). Although our study is limited to three patients, we found that they displayed many of the antibody sequence abnormalities reported by others (Fig. 1 and references 28–38). For example, mature naive B cells from SLE122 showed a decrease in V$_H$4 (10.4 vs. 30.4% in controls) and increased V$_H$3 (60.4 vs. 39.3% in controls) gene family representation (Fig. 1 B). SLE122 was also unusual in that IgH chains from both new emigrant and mature naive B cells displayed short CDR3 regions (Fig. 1, A and B). In this patient, the average IgH CDR3 length was 12.6 amino acids (aa) in new emigrant B cells compared with 14.1 aa in controls (P = 0.033) and 11.5 aa in mature naive B cells compared with 13.4 aa in controls (P = 0.003). We found no evidence for an increase in Ig light (IgL) chain receptor editing as determined by downstream Jκ/λ usage, but Vκ4–1 was overrepresented in SLE100 and SLE122 (Fig. 1, C and D, and references 28, 35). Despite these alterations in the Ig gene usage in individual patients, we found no consistent Ig gene repertoire or sequence abnormalities in the antibodies expressed by new emigrant and mature naive B cells from patients with SLE (Fig. 1 and Tables S1–S6).

Autoreactive antibodies fail to be removed at the transition between new emigrant and mature naive B cells in patients with SLE

To determine the frequency of autoreactive antibodies in new emigrant and mature naive B cells in SLE, we expressed antibodies cloned from single cells and tested them for autoreactivity by HEp-2 cell lysate ELISA and indirect immunofluorescence assay (IFA) on fixed HEp-2 cells (Fig. 2, Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20042251/DC1, and Tables S1–S6). The HEp-2 ELISA assay is a clinical test for ANA reactivity that uses HEp-2 cell lysate as the antigen (Fig. 2, A and B). Using this assay, we reported previously that 40.7% of new emigrant and 20.4% of mature naive B cells from healthy individuals express HEp-2–reactive antibodies (Fig. 2 and reference 13). Thus, healthy humans display a checkpoint in B cell development between the new emigrant and mature naive compartments that removes a substantial number of HEp-2–reactive antibodies (13). Antibodies from new emigrant B cells from SLE patients were not significantly different in HEp-2 ANA ELISA reactivity (40.7% in controls and 40.5–59.4% in SLE; P = 0.401) from antibodies of healthy donors (Fig. 2 A and reference 13). In contrast, antibodies from mature naive B cells from all three SLE patients differed from controls in that they retained high levels of HEp-2 ANA ELISA–reactive antibodies (41.5–50.0 compared with 20.4% in controls, P = 0.0003; P = 0.009 for SLE100, P = 0.004 for SLE101, and P = 0.031 for SLE122; Fig. 2 B and reference 13). We conclude that mature naive B cells in these three adolescent SLE patients are enriched in antibodies that react with HEp-2 cell lysates.

High affinity ANAs are a hallmark of SLE. To determine if HEp-2 ELISA–reactive antibodies from B cells of
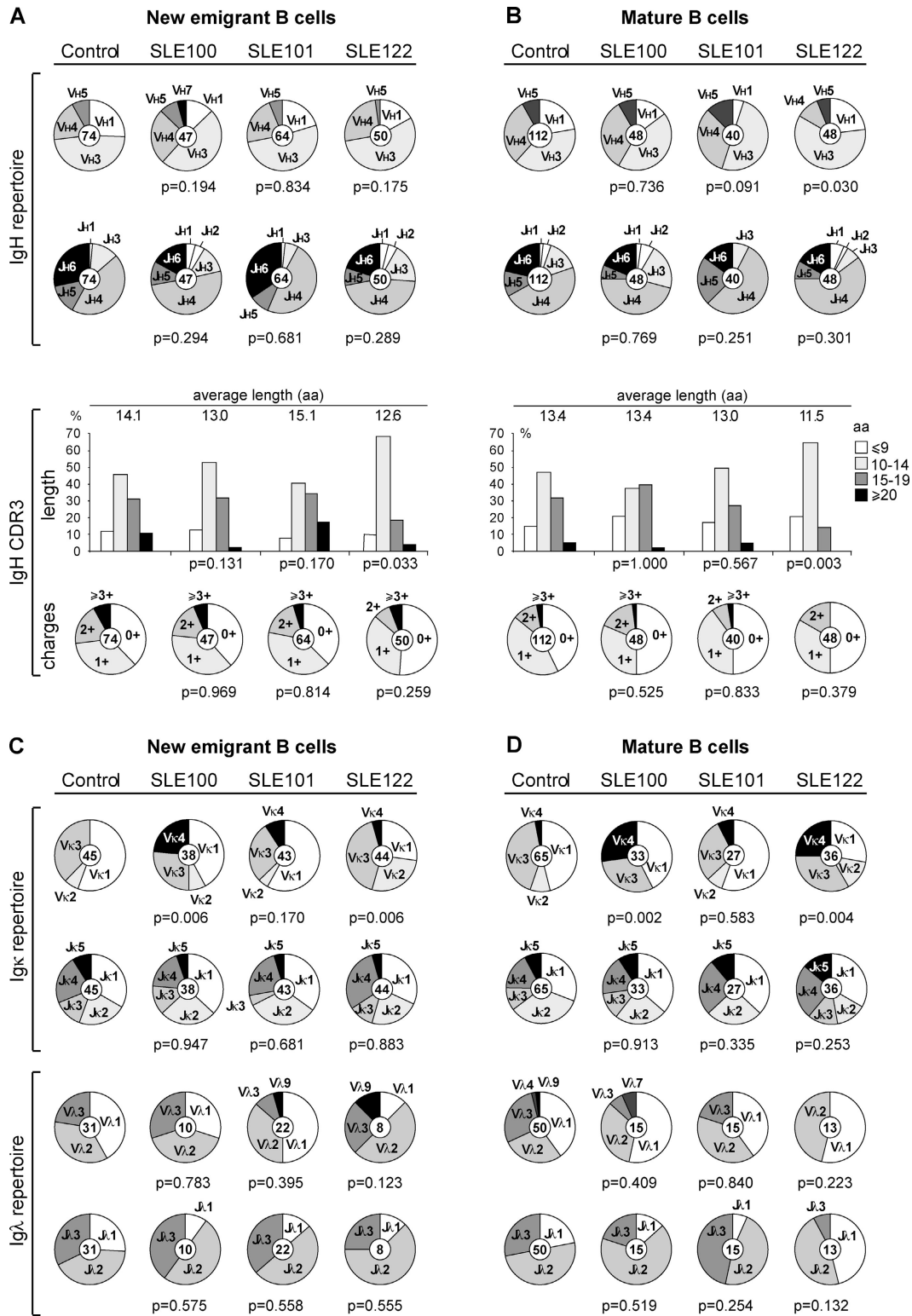
Figure 1.   IgH and IgL chain sequence features. IgH V and J gene repertoire, CDR3 length, and number of positively charged residues from new emigrant (A) and mature naive (B) B cells. Pie charts depict V_H and J_H family usage (top) and the proportion of IgH CDR3s with 0, 1, 2, or ≥3 positive charges (bottom). Bar graphs show frequencies of IgH CDR3s with 9 aa (white bars), 10–14 aa (light gray bars), 15–19 aa (dark gray bars), and ≥20 aa (black bars). The absolute number of sequences analyzed in each B cell compartment is indicated in the center of the pie charts. V and J family gene usage for Igκ and Igλ light chains from new emigrant (C) and mature naive (D) B cells. Values for controls in this and other figures were published previously and are shown here for comparison (13). p-values indicated below the charts are in comparison with control (reference 13).
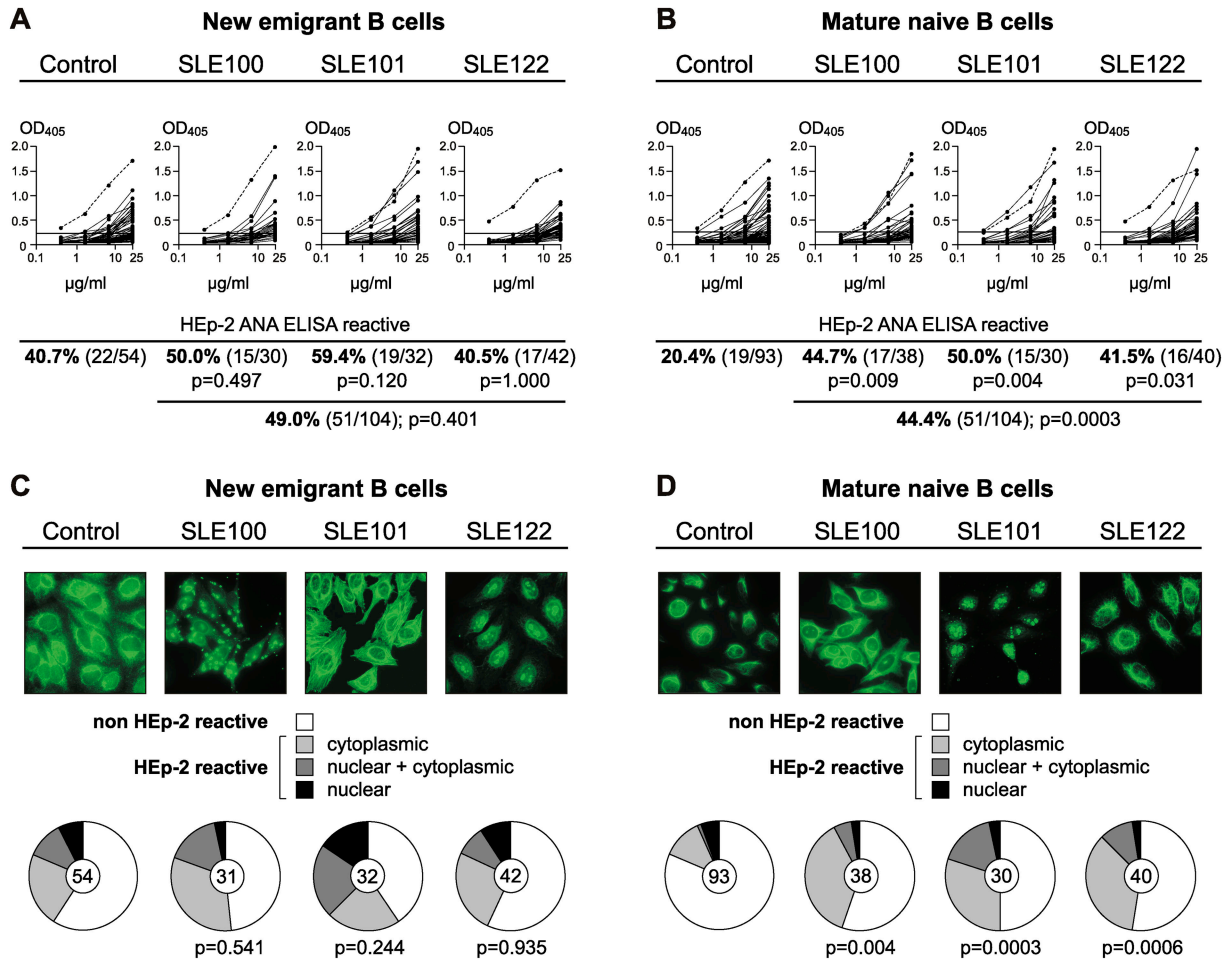
Figure 2. HEp–2 ELISA and immunofluorescence. HEp-2 ANA ELISA results for antibodies cloned from new emigrant (A) and (B) mature naive B cells from SLE patients. Dotted lines show ED38 positive control (references 13, 44). Horizontal line shows cut-off OD_{405} for positive reactivity. Staining patterns of antibodies found in new emigrant (C) and mature (D) antibodies by IFA (Fig. S2). For each patient, the frequency of HEp-2 reactive and nonreactive clones as measured by HEp-2 ANA ELISA (A and B) or IFA (C and D) is summarized in pie charts with the number of antibodies tested indicated in the centers. All p-values are in comparison to previously published controls from the same B cell compartment (reference 13).

SLE patients are specifically enriched for ANAs, we analyzed the subcellular staining pattern of these antibodies by IFA on HEp–2 cell–coated slides (Fig. 2, C and D, and Fig. S2). We found that, similar to healthy control antibodies, HEp–2–reactive antibodies from new emigrant B cells of SLE patients were not enriched in ANAs and only a small fraction of the self-reactive antibodies expressed by mature naive B cells from SLE patients recognize nuclear antigens as measured by IFA (Fig. 2 D). We conclude that in SLE, HEp–2–reactive antibodies fail to be removed in the peripheral checkpoint between the new emigrant and mature naive B cell compartments.

High numbers of polyreactive antibodies are present in mature naive B cells of patients with SLE

In addition to HEp–2 reactivity, we also measured the reactivity of the cloned antibodies to a defined set of antigens including ssDNA, dsDNA, insulin, and LPS (Fig. 3). In healthy individuals, 55% of newly arising human B cells in the bone marrow express antibodies that bind to at least two of these antigens and, therefore, are considered polyreactive (13). However, the majority of these potentially harmful antibodies are removed from the repertoire at the early immature B cell stage and only a small number of the new emigrant B cells that leave the bone marrow are weakly polyreactive (13). Therefore, we reasoned that increased numbers of polyreactive antibodies in the peripheral blood new emigrant B cell fraction of SLE patients would reflect a failure to remove potentially harmful autoantibodies during early B cell development in the bone marrow. Of the three patients tested, only SLE101 showed a significant increase in polyreactive antibodies in the new emigrant B cell compartment (51.5 vs. 7.4% in control; P < 0.0001; Fig. 3 A). Many of these antibodies (5/17) also differed qualitatively from the control polyreactive antibodies in that they displayed stronger levels of antigen binding in the ELISA (Fig. 3 A). Thus,
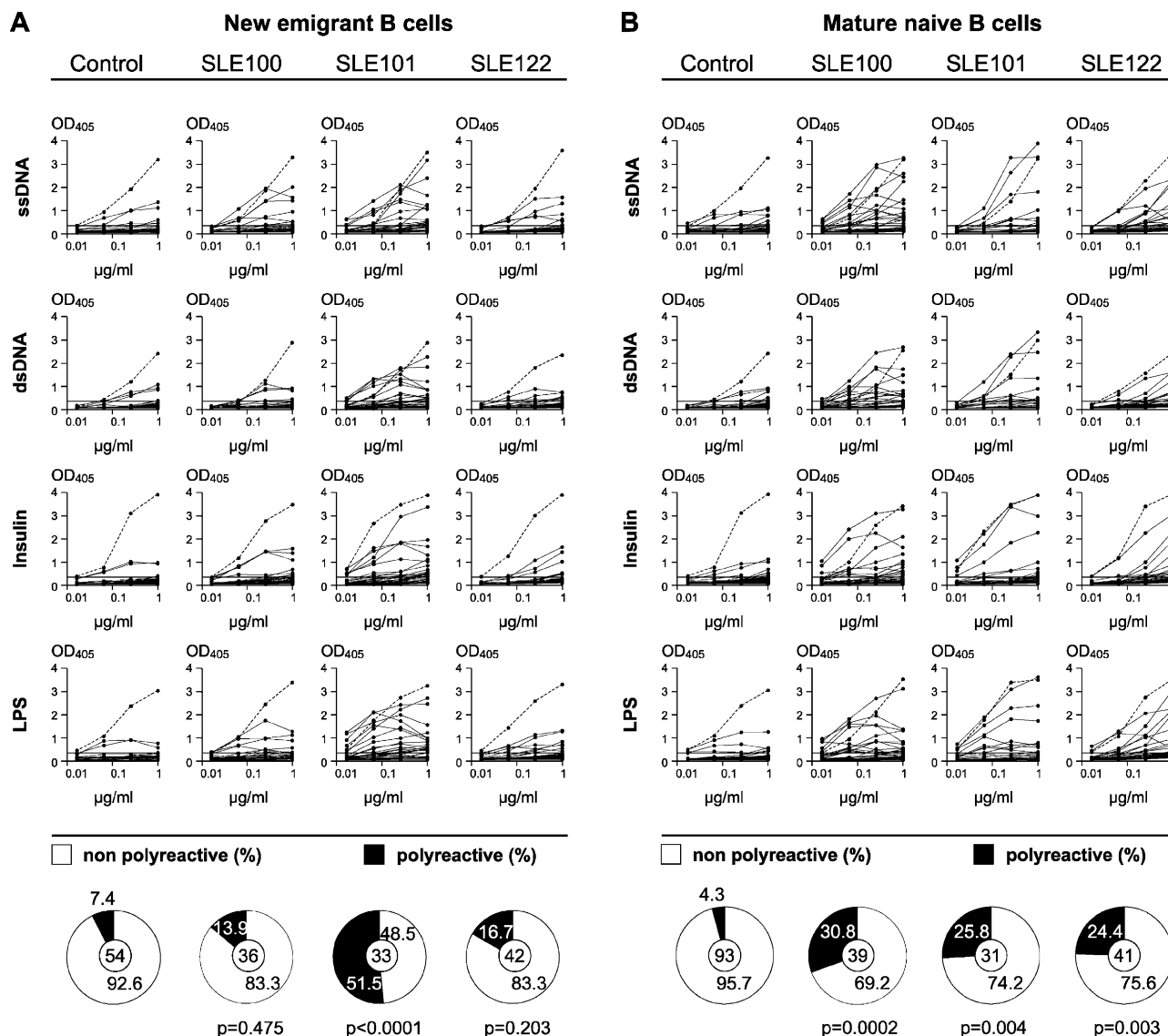
Figure 3. Polyreactivity ELISAs. Antibodies from (A) new emigrant and (B) mature naive B cells were tested by ELISA for reactivity with ssDNA, dsDNA, insulin, and LPS. Dotted lines show ED38 positive control (references 13, 44). Horizontal line shows cut-off OD_{405} for positive reactivity. The frequency of reactive clones in percent and the absolute number of reactive antibodies out of all tested antibodies are indicated below the graphs. p-values are in comparison to previously published controls from the same B cell compartment (reference 13).

the frequency and strength of reactivity of the polyreactive antibodies in the new emigrant B cell compartment of SLE101 is similar to that found in the early immature B cells in the bone marrow of healthy donors (55.2%; P = 0.804; reference 13). SLE100 and SLE122 also showed an increase in the number of polyreactive antibodies in the new emigrant B cell fraction, but this did not reach statistical significance (13.9 and 16.7%, respectively, vs. 7.4% in controls; Fig. 3 A). We conclude that SLE101 shows a significant defect in the early B cell tolerance checkpoint between the bone marrow and the periphery.

Significantly increased numbers of mature naive B cells from all three SLE patients expressed self-reactive antibodies as measured by HEp-2 reactivity (Fig. 2, B and D). To deter-

mine whether mature naive B cells were also enriched in polyreactive antibodies, we tested antibodies from all three patients in antigen-specific ELISAs (Fig. 3 B). We found high frequencies of polyreactive antibodies in the mature naive B cell compartment of all three patients when compared with healthy controls (controls: 4.3 vs. 30.8% for SLE100, P = 0.0002; 25.8% for SLE101, P = 0.004; 24.4% for SLE122, P = 0.003; P = 0.0001 for SLE combined; Fig. 3 B), and some of these antibodies demonstrated high levels of reactivity in ELISA, a feature that was not seen in mature naive B cells from healthy donors (Fig. 3 B and reference 13). In the case of SLE100 and SLE122, the level of polyreactivity in the mature naive compartment represents an increase when compared with the new emigrant compartment, whereas in
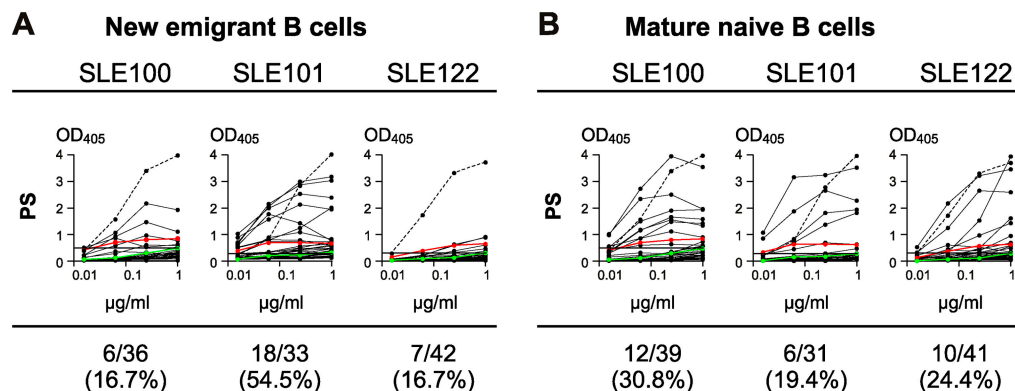
A New emigrant B cells

SLE100   SLE101   SLE122

6/36
(16.7%)

18/33
(54.5%)

7/42
(16.7%)

B Mature naive B cells

SLE100   SLE101   SLE122

12/39
(30.8%)

6/31
(19.4%)

10/41
(24.4%)

Figure 4. PS ELISA. Antibodies from (A) new emigrant and (B) mature naive B cells were tested by ELISA for reactivity with phosphatidylserine (PS). Dotted lines show ED38 positive control (references 13, 44). Horizontal line shows cut-off OD_{405} for positive reactivity as determined by comparison to the negative control antibody mGO53 (green line) and low positive control antibody eiJB40 (red line) that were included in the assay. The frequency of reactive clones in percent and the absolute number of reactive antibodies out of all tested antibodies are indicated below the graphs.

SLE101, the number of polyreactive clones decreased in the transition. We conclude that patients with SLE have an abnormal peripheral B cell tolerance checkpoint resulting in the expression of large numbers of self-reactive antibodies in the mature naive B cell compartment.

Phosphatidylserine (PS) reactive antibodies

Two of the three SLE patients (SLE100 and SLE101) had increased serum titers of antiphospholipid antibodies, whereas SLE122 did not (Table I and Supplemental Materials and methods). To test if the antibodies from naive B cells of SLE100 and SLE101 included antiphospholipid specificities, we tested all antibodies from new emigrant and mature B cells for reactivity against PS (Fig. 4 and Tables S1–S6). We found that polyreactive antibodies from all three patients were reactive with PS (Fig. 4 and Tables S1–S6). However, this finding was independent of increased serum autoantibody titers against PS as measured in SLE101 but not in SLE100 or SLE122 as part of the serology (Supplemental Materials and methods). We conclude that polyreactive antibodies enriched in naive B cells in SLE are also reactive with SLE-associated antigens.

DISCUSSION

Previous studies of the antibody repertoire expressed by peripheral B cells in SLE revealed enormous variability between patients (33). Studies of individual $CD19^+$ cells from SLE patients showed alterations in mutational activity in IgH and IgL genes, and alterations of Ig repertoire (28–33). The diverse nature of Ig repertoire alterations, including bias toward V_H3, V_H4-34, and $V_\kappa1$ gene family usage, has been demonstrated by a number of groups (34–38). Our Ig sequence analysis is consistent with these findings and, in combination with antigen-binding experiments, also establishes that the Ig gene repertoire is not predictive of a self-reactive antibody selection bias in SLE.

For example, V_H3 usage was reported to be increased in SLE (29), and we found this V_H gene family overrepresented in mature naive B cells in SLE122, but not in the other two patients. More importantly, we found no correlation between V_H3 and self-reactivity; only V_H4 showed a small relative enrichment in autoreactivity (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20042251/DC1). Autoreactivity and polyreactivity are associated with positively charged and long heavy chain CDR3s (13, 39–44). Consistent with this observation, we found a statistically significant correlation between the number of positive charges in IgH CDR3 and polyreactivity (Fig. S3). A consistent finding among all three SLE patients was a significant increase in autoreactive and polyreactive antibodies in the mature naive B cell compartment. However, neither the number of IgH CDR3-positive charges nor their lengths or specific Ig gene usage was sufficient to predict polyreactivity in SLE patients or healthy volunteers (reference 13 and Fig. S3). We conclude that the diversity of the human antibody repertoire precludes the use of sequence analysis alone as an indicator of either self-reactivity or abnormalities in self-reactive B cell selection.

How might the diverse genetic defects associated with SLE lead to a break in early B cell tolerance checkpoints and an accumulation of autoreactive B cells in the mature naive B cell compartment? Experiments in mice indicate that central B cell tolerance is mediated by three mechanisms: receptor editing, deletion, and anergy (14–16, 45). B cell signaling strength and the physical nature of the self-antigen (soluble vs. membrane bound) are important determinants in the choice between editing, deletion, and anergy (46, 47). A number of genes that regulate B cell receptor signaling thresholds have been implicated in SLE, and any of these might impact on selection to produce a defect in early immature B cell tolerance (4, 48). A defect in either of these mechanisms could account for the high number of autoreactive antibodies in new emigrant B cells of SLE101.

In all three SLE patients, we observed a striking increase in autoreactive antibodies in mature naive B cells, and there is little known about the mechanisms that govern the tolerance

checkpoint between new emigrant and mature naive B cell compartments. Indeed, the roles of positive and negative selection in this transition are still debated (20–23), but B cells with autoreactive receptors are deleted between the new emigrant and mature naive B cell stage in mice (49, 50). Our experiments show that the central and peripheral B cell tolerance checkpoints are regulated independently in humans because the defect in SLE101 was partially corrected in the transition to the mature naive B cell compartment, whereas it was exacerbated in SLE100 and SLE122. These differences are likely to reflect the individual patients genotype and the relative contributions of SLE susceptibility genes such as signal transducers, coreceptors, cytokines, and regulators of antigen clearance that govern B cell tolerance checkpoints (4–8).

Do high numbers of autoreactive antibodies in the mature naive B cell compartment of SLE patients predispose to the development of the disease and the generation of clinical symptoms? Individuals that develop SLE have high titers of circulating autoantibodies long before they develop clinical symptoms of the disease (51). Although the antiself-antibodies expressed by naive B cells in SLE may not be directly pathogenic, they can readily be mutated to higher affinity and switch to downstream isotypes once a B cell is activated and antinuclear specificities might be selected or expanded during this maturation process (17, 52–55).

All three SLE patients showed high frequencies of antibodies that recognized DNA, which is part of cellular debris (56–58). In this context, DNA can trigger B cell responses by simultaneously activating B cell antigen receptor and Toll like receptor signaling pathways leading to potent B cell responses, including antibody production and further B cell maturation (59, 60). Mutations that interfere with physiologic pathways governing antigen clearance would be particularly likely to induce autoantibody responses in individuals such as the SLE patients described here because they show large numbers of polyreactive B cells that would be activated by DNA containing antigens. In healthy individuals, inhibitory mechanisms such as those mediated by inhibitory receptors, for example FcγRIIb, may limit activation of the small numbers of autoreactive B cells present in the mature B cell compartment (61). We speculate that these protective mechanisms are overwhelmed in the presence of large numbers of mature naive self-reactive B cells that produce polyreactive antibodies in SLE. Consistent with this idea, transgenic expression of BAFF in mice produces an SLE-like disease by increasing the number of autoreactive B cells that survive the transition between the new emigrant and mature naive B cell compartment (62–67).

In conclusion, our experiments show that patients with SLE have a previously unappreciated defect in early B cell tolerance checkpoints, leading to accumulation of large numbers of autoreactive B cells in the mature naive B cell compartment. This feature of the disease may explain why SLE patients suffer frequent relapses after conventional treatment and suggests that restoring tolerance in mature naive B cells might be an effective approach to more enduring therapies.

MATERIALS AND METHODS

Patient samples and single B cell sorting. The study was performed in accordance with IRB-reviewed protocols of the UT Southwestern Medical Center (IRB no. 0199-017) and The Rockefeller University (HWA-0518-1003), and all samples were obtained after signed informed consent. Peripheral blood samples from three untreated SLE patients were obtained at the Division of Pediatric Rheumatology of UT Southwestern Medical Center and shipped overnight. Peripheral blood from healthy controls was obtained at The Rockefeller University and kept overnight on a shaker at room temperature. SLE and control blood samples were processed in parallel for comparison and FACS settings. Control data shown are from previously published healthy controls (13). Single B cell isolation was performed as described previously (13). In brief, B cells were preenriched by incubation of peripheral blood with RossetteSep according to the manufacturer's instructions (Stem Cell Technologies, Inc.) followed by Ficoll-Hypaque (Amersham Biosciences) gradient centrifugation. Enriched B cells were stained with anti–human CD10-PE (BD Biosciences) before separation with anti-PE magnetic bead columns (Miltenyi Biotec). CD10-enriched and CD10-depleted B cell fractions were stained with anti–human CD19-APC, anti–human CD10-PE, anti–human IgM-biotin, and anti–human CD27-FITC antibodies (BD Biosciences), and biotinylated anti–human IgM antibody was revealed using streptavidin-PeCy7 (Caltag Laboratories). New emigrant B cells (CD19$^+$CD10$^+$IgM$^+$CD27$^-$) were isolated from the CD10-enriched B cell fraction and mature naive B cells (CD19$^+$CD10$^-$IgM$^+$CD27$^-$) were isolated from the CD10-depleted B cell fraction (Fig. S1). Single B cells were purified by FACS into 96-well plates containing 4 μl of 0.5 × PBS, 10 mM DTT, 8 U RNAsin (Promega), and 3 U Prime RNase Inhibitor (Eppendorf) using a FACSvantage (Becton Dickinson). All samples were immediately frozen on dry ice and stored at −70°C.

PCR amplification and expression vector cloning. Single cell cDNA was synthesized in the original sort plates in a total volume of 14.5 μl (4 μl sort lysis solution + 10.5 μl RT-PCR reaction mix). RT-PCR reaction mix contained 150 ng random hexamer primer (pd[N]$_6$; Amersham Biosciences), 0.5% vol/vol Igepal CA-630 (NP40; Sigma-Aldrich), 7 mM DTT, 4 U RNAsin (Promega), 7.5 U Prime RNase Inhibitor (Eppendorf), 1.2 mM each dNTP, and 50 U Superscript II RT (Invitrogen), and cDNA was synthesized at 42°C for 55 min. Individual IgH (μ) and IgL chain κ or λ gene rearrangements were amplified in two successive rounds of PCR (50 cycles each) before cloning into human Igγ1, Igκ, or Igλ expression vectors using primers that include restriction sites as described previously (13). Primer sequences were as described in Table S7, available at http://www.jem.org/cgi/content/full/jem.20042251/DC1. Individual Ig genes were amplified with 3.5 μl cDNA or first PCR product in 40 μl of total reaction volume with 7.5 p.m. 5′ and 3′ primers or primer mixes, 312.5-μM each dNTP and 1 U Hotstar Taq DNA polymerase (QIAGEN). Ig genes were analyzed using National Center for Biotechnology Information IgBLAST.

Antibody production and purification. Antibodies were expressed in vitro as described previously (13). In brief, human embryonic kidney fibroblast 293A cells were cotransfected with 12.5 μg/ml of IgH and IgL chain encoding plasmid DNA by calcium phosphate precipitation. Cells were washed with serum-free DMEM 8–12 h after transfection and thereafter cultured in DMEM supplemented with 1% Nutridoma SP (Roche). Supernatants were collected after 8 d of culture. Antibodies were purified by binding to protein G–Sepharose (Amersham Biosciences) and elution with 0.1 M glycine buffer, pH = 3. Eluted antibodies were kept at physiologic pH. Antibody concentrations in supernatants and of purified antibodies were determined by anti–human IgG1 ELISA using human monoclonal IgG1 as standard (Sigma-Aldrich).

ELISA and IFA. ELISAs were performed as described previously (13). In brief, tissue culture supernatants were adjusted to a starting antibody concentration of 1 μg/ml for polyreactivity ELISAs and used at three subsequent 1:4 dilutions in PBS. Specific antigens were coated on microtiter plates (Easy-

wash Polystyrene Plates; Costar) at 10 μg/ml for ssDNA, dsDNA, and LPS (Sigma-Aldrich) or 5 μg/ml for recombinant human insulin (Sigma-Aldrich). For PS ELISAs, plates were coated with 50 μl 10 μg/ml L-α-phosphatidyl-L-serine, dioleoyl (Sigma-Aldrich) in ethanol and plates were allowed to air dry. Samples from SLE patients were considered negative if the OD_{405} did not exceed a threshold value as indicated in each graph at any of the four dilutions in at least three independent experiments (Tables S1–S6). Threshold values were set in all assays using antibodies from healthy donors and included the negative control antibodies mGO13, mGO53, low positive control antibody eiJB40, and high polyreactive antibody ED38 (13, 44). HEp-2 ELISAs were performed on QUANTA Lite ANA ELISA plates (INOVA Diagnostics) and coated with HEp-2 cell lysates. Purified antibodies were used at a concentration of 25 μg/ml with three subsequent 1:4 dilutions in PBS. The threshold OD_{405} below which samples were considered negative was 0.3. Positive and negative controls included sera from patients and healthy individuals (INOVA Diagnostics) as well as ED38 (13) and were included in every experiment. All ELISAs were developed with horseradish peroxidase–labeled goat anti–human IgG Fc Ab (Jackson ImmunoResearch Laboratories) and horseradish peroxidase substrate (Bio-Rad Laboratories). OD_{405} was measured on a microplate reader (Molecular Devices).

IFAs were performed as described previously (13). In brief, HEp-2 cell coated slides (Bion Enterprises, Ltd.) were incubated at room temperature with purified antibodies at 25–150 μg/ml for 30 min, washed in PBS and visualized with FITC anti–human Ig by fluorescence microscopy. Controls included ED38 (13) and positive and negative sera (Bion Enterprises, Ltd. and Fig. S2).

Statistics. p-values for Ig gene repertoire analyses, analysis of positive charges in IgH CDR3, and antibody reactivity were calculated by 2×2 or 2×5 Fisher's Exact test or χ^2 test. p-values for IgH CDR3 length were calculated by Student's t test.

Online supplemental material. Supplemental Materials and methods describes clinical information for SLE100, SLE101, and SLE122. Tables S1–S6 show IgH and IgL chain characteristics and antibody reactivities with dsDNA, ssDNA, LPS, insulin, and PS from new emigrant and mature naive B cells of SLE100, SLE101, and SLE122. Table S7 shows PCR primer sequences. Fig. S1 shows representative FACS profiles of CD10-enriched and CD10-depleted B cell fractions used for single cell purification. Fig. S2 shows representative IFA staining patterns of representative nonreactive SLE antibodies and positive and negative controls. Fig. S3 shows V_H usage and IgH CDR3 length for polyreactive versus nonpolyreactive antibodies from B cells of SLE patients. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042251/DC1.

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