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Checkpoints for Autoreactive B Cells in the Peripheral Blood of Lupus Patients Assessed by Flow Cytometry

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Abstract

Objective—Antinuclear antibodies (ANAs) are diagnostic in several autoimmune disorders, yet the failure to achieve B cell tolerance in these diseases is still poorly understood. Although secreted ANAs detected by an indirect immunofluorescence assay are the gold standard for autoreactivity, there has been no convenient assay with which to measure the frequency of circulating B cells that recognize nuclear antigens (ANA + B cells) in patients. The aim of this study was to generate an assay to easily identify these B cells and to examine its utility in a study of autoreactive B cells in systemic lupus erythematosus (SLE).

Methods—We developed and validated a novel flow cytometry–based assay that identifies ANA + B cells using biotinylated nuclear extracts, and utilized it to examine B cell tolerance checkpoints in peripheral blood mononuclear cells obtained from SLE patients and healthy controls.

Result—We observed progressive selection against ANA + B cells as they matured from transitional to naive to CD27 + IgD– and CD27 + IgD + memory cells in both healthy subjects and SLE patients; however, ANA + naive B cells in SLE patients were not anergized to the same extent as in healthy individuals. We also showed that anergy induction is restored in SLE patients treated with belimumab, an inhibitor of BAFF.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Diamond had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conclusion—This assay will enable studies of large populations to identify potential genetic or environmental factors affecting B cell tolerance checkpoints in healthy subjects and patients with autoimmune disease and permit monitoring of the B cell response to therapeutic interventions.

Autoreactivity arises as a consequence of creating a diverse repertoire of B cells but is held in check by processes that result in deletion, receptor editing, or anergy at multiple junctures prior to maturation to the naive B cell stage. Approximately 75% of early immature B cells in healthy individuals are self-reactive, but both antinuclear reactivity and polyreactivity to single-stranded DNA, double-stranded DNA (dsDNA), lipopolysaccharide, and/or insulin are removed as B cells transition from immature to transitional to naive stages of development (1). In systemic autoimmune rheumatic diseases, including systemic lupus erythematosus (SLE), Sjogren's syndrome, systemic sclerosis, idiopathic inflammatory myopathies, and connective tissue disease, the detection of antinuclear antibodies (ANAs) in the serum of patients by indirect immunofluorescence staining of HEp-2 cells is important diagnostically (2). It is not, however, fully understood which checkpoints are breached, leading to ANA production.

Most of our current knowledge regarding the regulation of the B cell receptor repertoire in humans derives from the analysis of cloned recombinant antibodies reconstituted from single B cells and subsequently analyzed for their antigenic reactivity. A failure in central tolerance of polyreactive B cells in the bone marrow at the immature B cell stage and a failure in peripheral tolerance of ANA+ B cells and polyreactive B cells in the blood at the transitional-to-naive B cell checkpoint was observed in a study of recombinant antibodies derived from 3 SLE patients with active disease who were not yet receiving therapy (3,4). Although this and similar studies have generated important information regarding tolerance checkpoints for autoreactive B cells, the technology is extremely labor-intensive and not suitable for the analysis of large numbers of subjects (5,6).

We developed a novel flow cytometry method that easily identifies individual ANA+ B cells and applied this method to investigate B cell tolerance checkpoints in SLE patients and healthy control subjects. Both SLE patients and healthy controls demonstrated a reduction in the frequency of ANA+ B cells between the transitional/naive and naive/memory cell checkpoints. However, we observed that SLE patients demonstrate a defect in the induction of anergy in ANA+ B cells within the naive B cell compartment. Our analysis of B cells from belimumab-treated SLE patients showed that BAFF blockade restores tolerance by anergy in ANA+ B cells and demonstrated the importance of anergy as a mechanism of B cell tolerance.

PATIENTS AND METHODS

Patients and healthy donors

A total of 46 SLE patients and 33 healthy control subjects were recruited. Several of the control subjects were recruited from the Genotype and Phenotype Registry at the Feinstein Institute for Medical Research. At the time of the blood draw, all SLE patients were assessed for disease activity using the SLE Disease Activity Index (SLEDAI) (7). Nine SLE patients had been receiving belimumab treatment for a mean $\pm$ SD of 64.8 ± 22.5 months (minimum

of 43 months and maximum of 96 months), while the remaining 37 SLE patients had never been treated with belimumab. Blood samples were collected from SLE patients and healthy control subjects according to protocols approved by the Northwell Health institutional review board, and written informed consent was obtained from all participants.

Preparation of biotinylated nuclear antigens

HeLa cells were grown to confluence in a T75 flask, and nuclei were isolated using a Nuclei EZ lysis kit, according to the protocol of the manufacturer (Sigma-Aldrich). The nuclei were washed with phosphate buffered saline (PBS) and pelleted at 500g for 5 minutes. The pellet was resuspended in 3 ml of PBS containing a cOmplete Mini Protease Inhibitor tablet (Roche), split into 2 tubes, and vortexed vigorously on a Disruptor Genie with 150 μ l of 0.5-mm glass beads (Scientific Industries) in each tube for 1 hour at 4°C. The nuclear extract was then spun at 14,000 revolutions per minute for 5 minutes and the supernatant labeled using an EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo Scientific). The solution was dialyzed in PBS for 24 hours at 4°C, and aliquots were stored at –80°C.

Flow cytometry for analysis of human B cells and single-cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by density-gradient centrifugation using Ficoll-Paque (GE Healthcare) and stored in 10% DMSO and 90% fetal bovine serum (FBS) at –80°C. Cryopreserved PBMCs were thawed, washed, and incubated in 5% FBS in Hanks' balanced salt solution (HBSS) for 30 minutes at 37°C. PBMCs (5×10^6) were then spun down and resuspended in 100 μ l of 1.5% nonfat dry milk (Fisher Scientific) in HBSS with 10 μ l of biotinylated nuclear extract and incubated on ice for 30 minutes. The cells were spun down, resuspended, and stained for 15 minutes on ice with a cocktail of monoclonal antibodies: fluorescein isothiocyanate–conjugated anti-IgD (clone IA6-2; BD Biosciences), phycoerythrin (PE)–conjugated anti-CD27 (clone CLB-27/1; Invitrogen), PE–Texas Red–conjugated anti-CD38 (clone HIT2; Invitrogen), and Alexa Fluor 700–conjugated anti-CD3 (clone HIT3a), Alexa Fluor 700–conjugated anti-CD14 (clone M5E2), Alexa Fluor 700–conjugated anti-CD16 (clone 3G8), PerCP–Cy5.5–conjugated anti-IgM (clone MHM-88), PE–Cy7–conjugated anti-CD10 (clone HI10a), allophycocyanin-conjugated anti-CD19 (clone HIB19), and BV421-conjugated streptavidin (all from BioLegend). For analysis, $3\text{--}5 \times 10^5$ events were collected for each sample using an LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

Analysis of HeLa nuclear extract composition

For DNA analysis, biotinylated HeLa nuclear extracts were loaded on a 1% agarose gel (Invitrogen) and electrophoresed at 90 volts for 2 hours. For protein analysis, the nuclear extracts were resolved on a 4–12% Bis-Tris NuPAGE mini gel (Invitrogen) in NuPAGE MES SDS Running Buffer (Invitrogen) and visualized using SimplyBlue SafeStain (Invitrogen). For detection of the Sm antigen, proteins were transferred to a 0.45- μ m PVDF membrane (Immobilon FL), and the membrane was blocked in 50% LI-COR Blocking Buffer (PBS) for 1 hour and incubated with an anti-Sm monoclonal antibody (clone Y12; Thermo Fisher Scientific) at 1:200 in blocking buffer with 0.2% Tween for 2 hours. The membrane was washed and then incubated with IRDye 680RD–conjugated anti-mouse IgG

(LI-COR) at 1:5,000 in blocking buffer with 0.2% Tween and 0.01% sodium dodecyl sulfate for 1 hour.

For detection of La/SSB, proteins were transferred to 0.2- μ m Novex nitrocellulose membrane (Invitrogen), and the membrane was blocked overnight at 4°C in LI-COR blocking buffer and incubated with a 1:5,000 dilution of rabbit anti-human SSB (Bethyl Laboratories) in blocking buffer with 0.2% Tween for 4 hours. After washing, the membrane was incubated with IRDye 800CW-conjugated anti-rabbit IgG (LI-COR) at 1:15,000 in blocking buffer with 0.2% Tween for 1 hour. Unless otherwise indicated, all incubations were performed at room temperature, and all washes were in PBS–0.1% Tween except for the final wash in PBS.

Single-cell reverse transcription–polymerase chain reaction (PCR), immunoglobulin gene amplification, and expression vector cloning

ANA + and ANA– transitional and naive B cells were individually sorted onto 96-well PCR plates containing 4 μ l of 0.5 $\times$ PBS, 10 mM dithiothreitol (DTT), 11 units Prime RNase Inhibitor (Qiagen), using a FACSARIA sorter (Becton Dickinson), immediately frozen on dry ice, and stored at –80°C. Complementary DNA was synthesized from single sorted cells in a total volume of 14.5 μ l containing 150 ng random hexamer primer (pd[N]6; GE Healthcare Life Sciences), 0.5% volume/volume Igepal CA-630 (Nonidet P40; Sigma-Aldrich), 7 mM DTT, 11.5 units Prime RNase Inhibitor, 1.2 mM each dNTP (USB), and 50 units Superscript III reverse transcriptase (Invitrogen), at 42°C for 55 minutes. Immunoglobulin heavy chain (IgH) (μ only) and immunoglobulin light chain (IgL) (κ and λ) transcripts were amplified in 2 rounds of PCR using a combination of primers that amplify all of the Ig μ , Ig κ , and Ig λ gene families, as previously described (1). PCR efficiencies for obtaining both heavy chain and light chain transcripts ranged from 70% to 80%. The PCR products were subjected to exoSAP treatment (USB Biochemicals) and sequenced (Keck DNA Sequencing Facility, Yale University). The sequences were analyzed using BLAST (<http://www.ncbi.nlm.nih.gov/igblast>). Complete in-frame sequences of Ig μ and Ig κ or Ig λ transcripts were reamplified using family-specific primers that include restriction sites, digested with appropriate restriction enzymes (New England Biolabs), and cloned into human Ig γ 1, Ig κ , or Ig λ expression vectors, as described previously (1). The clones were screened by PCR, and plasmid DNA was prepared (Qiagen Miniprep kit) and resequenced to confirm fidelity to the original PCR product.

Antibody production and purification

Human embryonic kidney fibroblast cells (HEK 293) were cotransfected with 5 μ g each of IgH and IgL encoding plasmid DNA, using Lipofectamine 2000 (Life Technologies), and the cells were cultured in serum-free medium (HyClone Transfx HEK-293) for 6 days. Antibodies in the culture supernatants were bound to protein G–Sepharose (GE Life Sciences), eluted with 0.1M glycine buffer (pH 3.5), and stored in PBS at 4°C. Antibody concentrations in culture supernatants, as well as concentrations of purified antibodies, were determined by anti-human IgG1 enzyme-linked immunosorbent assay (ELISA), using human IgG1 monoclonal antibody as standard (SouthernBiotech).

ANA ELISA

An ELISA was performed to assess the reactivity of the cloned antibodies to nuclear antigens. Quanta Lite ANA ELISA plates (Inova Diagnostics) were blocked with 3% FBS–PBS and incubated with purified antibodies at 10 µg/ml concentration. Alkaline phosphatase–coupled IgG was added, followed by phosphatase substrate tablets (Sigma). All incubations were carried out for 1 hour at 37°C, and the plates were washed with PBS–0.05% Tween.

Immunofluorescence assay (IFA)

HEp-2 cell–coated slides (Bion Enterprises) were incubated with purified antibodies at 10 µg/ml for 30 minutes at room temperature, followed by incubation with Alexa Fluor 495–conjugated anti-human IgG (1:350 dilution) for 30 minutes. The slides were washed 3 × with PBS, mounted, and visualized by fluorescence microscopy (Zeiss).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (version 5.0d). Frequencies of ANA+ B cell populations were compared by 2-tailed Mann-Whitney U test. Correlations were calculated using Spearman's rank correlation test. Clinical group comparisons for age, prednisone dose, and SLEDAI scores were analyzed by Student's *t*-test. Analyses for ethnicity, sex, medication use, serology, and hypocomplementemia were performed using the chi-square test (IBM SPSS Statistics 21).

RESULTS

Identification of ANA1 B cells by flow cytometry

Our goal was to generate a flow cytometry–based method that allows a comprehensive view of the ANA+ B cell compartment to identify ANA+ B cells that are embargoed from becoming antibody-secreting plasma cells (ASCs) in healthy control subjects, and ANA+ B cells that have become dysregulated in patients with autoimmune disease. We produced antigenic fragments of nuclear extract by mechanically disrupting HeLa cell nuclei, which contained both DNA and some of the major protein targets previously described for ANAs, including Sm antigen and La/SSB (8) (Figure 1A). All of the major SLE protein antigens—histone, Sm antigen, Ro/SSA, and La/SSB—were biotinylated (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39710/abstract>). DNA was also biotinylated or present bound to biotinylated proteins in the extract (see Supplementary Figure 1). The biotinylated nuclear extract was incubated with human PBMCs. Fluorochrome-tagged streptavidin and B cell subset–specific markers detected and phenotyped B cells binding nuclear antigen fragments. The biotinylated extract showed specific staining of B cells compared with non-B cells (Figure 1B).

To prove that ANA+ B cells identified by flow cytometry bear receptors that recognize nuclear antigens, we performed single-cell cloning of immunoglobulins from equal numbers of sorted ANA+ and ANA– transitional and naive B cells derived from 3 control subjects and 1 SLE patient and tested their reactivity by standard clinical IFA on fixed HEp-2 cells

(Figures 1B and C). We analyzed the binding of 130 recombinant antibodies and observed that ~ 82% from ANA+ B cells displayed a nuclear or nuclear/cytoplasmic staining pattern by IFA at 10 $\mu\text{g/ml}$, while most from ANA– B cells did not (Figure 1C and Table 1). The flow cytometry–based assay was both sensitive and specific for autoreactivity (82% for both) (Table 1). We further confirmed the specificity of a subset of these antibodies using a commercial ANA ELISA that measures binding to HEp-2 nuclear antigens (Figure 1D). There were only 2 false-negative results by ELISA that were also false-negative by IFA. The 1 false-positive result by ELISA was also false-positive by IFA. Thus, we demonstrated that this novel ANA flow cytometry–based assay allows accurate analysis of the ANA + B cell repertoire.

Removal of ANA1 B cells at distinct peripheral B cell checkpoints in SLE patients and healthy controls

In order to track peripheral B cell tolerance checkpoints, we analyzed the frequencies of ANA+ B cells within the transitional (CD27–IgD+CD38^{high}CD10^{high}), naive (CD27–IgD+CD38^{intermediate}CD10^{low/-}), CD27+ memory, and double-negative (CD27–IgD–) compartments in SLE patients and age-matched healthy controls. The characteristics of the subjects are shown in Table 2. A group of SLE patients treated with belimumab was analyzed independently. In both SLE patients and healthy controls, the frequency of ANA+ reactivity was greatest in the transitional compartment and declined as B cells matured into naive and CD27+IgD+ or CD27+IgD– memory cells (Figure 2 and Table 3). In addition, the frequency of ANA+ B cells was diminished in the double-negative B cell compartment relative to that in the naive pool in both SLE patients and healthy controls; the percentage of ANA+ cells within the population of double-negative B cells was similar in both groups (Figure 2 and Table 3). Overall, SLE patients had lower proportions of ANA+ non-memory and classic CD27+IgD– memory cells compared with control subjects (Figure 2 and Table 3). We did not analyze CD27++ plasma cells, because our samples were cryopreserved, which markedly reduced the number of plasma cells.

In summary, this analysis revealed the removal of ANA+ B cells in both healthy controls and SLE patients at distinct checkpoints: between transitional and naive B cells, between naive and CD27+IgD+ memory B cells, and between naive and CD27+IgD– memory B cells. Analysis of total B cell populations showed that SLE patients exhibited similar frequencies of naive and CD27+IgD– memory cells, fewer CD27+IgD+ memory cells, and more transitional and double-negative B cells compared with healthy control subjects (see Supplementary Figure 2 and Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39710/abstract>).

Dysregulated anergy induction in autoreactive B cells in SLE patients

The induction of B cell anergy is an important mechanism of peripheral tolerance, serving to censor autoreactive B cells that have escaped deletion or receptor editing. Because peripheral tolerance checkpoints appeared to be intact in SLE at the stages mentioned above (Figure 2), and because most ANA + B cells were naive in both SLE patients (mean $\pm$ SD $70.0 \pm 13.5\%$) and healthy controls ($70.0 \pm 16.2\%$) (Figure 3A), we scrutinized the transitional/naive checkpoint for evidence of anergy. Anergic B cells were initially described

in healthy individuals as a small population of functionally attenuated, predominantly autoreactive mature naive B cells that expressed only IgD, not IgM (9). Later analyses showed that ~15–30% of naive B cells with reduced surface expression of IgM (IgM^{low}) were anergic (9,10). Another distinct anergic phenotype was identified as CD21^{low}CD27[–] but contained predominantly somatically mutated B cells in both healthy controls and SLE patients; this compartment was small in healthy individuals (11).

We used a gating strategy that identified anergic naive B cells as IgM^{low}IgD⁺CD27[–] (9). ANA + B cell reactivity was enriched in the IgM^{low} naive subset compared with IgM^{intermediate/high} naive B cells in both healthy controls ($P < 0.0001$) and SLE patients ($P = 0.0021$) (Table 3). Both the proportion of ANA + B cells within the IgM^{low} population (Figures 3B and C and Table 3) and the proportion of ANA + naive B cells that was IgM^{low} (results not shown) were significantly diminished in SLE patients compared with healthy controls ($P = 0.0109$ and $P = 0.0028$, respectively). Moreover, we observed a decreased frequency of IgM^{low} cells within the total B cell population in SLE patients (see Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39710/abstract>).

We substantiated our characterization of anergic B cells in several ways. First, to validate our gating strategy for IgM^{low} B cells, we explored an alternate scheme in a subset of samples, using total B cells instead of non-B cells, to set the gate for IgM^{low} B cells (see Supplementary Figure 3A, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39710/abstract>). This strategy similarly led to a significant difference in the frequency of anergic ANA + naive B cells between healthy controls and SLE patients ($P = 0.0062$) (see Supplementary Figure 3B). Next, we determined the stability of the anergic profile by examining blood samples obtained from 3 SLE patients at 2 time points. We observed little fluctuation in the frequency of anergic ANA + naive B cells over time. At ~2 month intervals, the frequencies changed from 4% to 2.9%, 2.3% to 0.4%, and 2.3% to 1.0% in the respective patients. Finally, we compared the responsiveness of IgM^{low} and IgM^{intermediate/high} naive B cells from healthy controls to B cell receptor crosslinking using intracellular calcium flux measurements (9,10). IgM^{low} cells were indeed hyporesponsive to stimulation through surface IgD (see Supplementary Figure 4, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39710/abstract>).

Restoration of the anergic phenotype in the naive autoreactive B cell population with BAFF blockade

Nine SLE patients treated with belimumab were studied (Table 2). Belimumab is an antibody to BAFF and is intended to reduce B cell autoreactivity. In mice, autoreactive B cells have an increased requirement for BAFF to enable maturation to immunocompetence (12). A lower frequency of ANA+ B cells was observed as B cells moved from transitional to naive to CD27⁺IgD[–] memory cells, similar to the decline observed in healthy controls and other SLE patients (Figure 2 and Table 3). A slightly greater proportion of ANA+ B cells in belimumab-treated SLE patients were transitional cells compared with the proportion in other SLE patients (mean $\pm$ SD 20.7 $\pm$ 15.6% versus 13.1 $\pm$ 12.8%), but this

difference did not reach statistical significance. A smaller proportion of ANA+ B cells were naive ($56.2 \pm 12.1\%$ in belimumab-treated patients versus $70.0 \pm 13.5\%$ in other SLE patients; $P=0.0079$) (Figure 3A). Most importantly, belimumab-treated SLE patients had a significantly greater frequency of anergic IgM^{low} B cells within the ANA+ naive B cell pool than other SLE patients, comparable with the frequency in healthy controls (Figures 3B and C and Table 3).

Possible but unlikely confounding factors contributing to the increased frequency of anergic ANA+ B cells in belimumab-treated SLE patients included age, ethnicity, and duration of disease. The belimumab-treated SLE patients were slightly older, had longer disease duration, were more often Hispanic, and less often African Americans (Table 2). The mean SLEDAI scores and prednisone doses were comparable between the 2 groups of SLE patients, but the SDs were large (Table 2).

Importantly, we observed significant inverse correlations between the frequency of anergic ANA+ B cells and SLEDAI scores ($r=-0.303$, $P=0.041$) when all SLE patients were combined, suggesting that more intact anergy correlates with less disease activity. In addition, we observed a reduced frequency of anergic ANA+ naive B cells in the combined SLE patients with high anti-dsDNA titers (mean $\pm$ SD $7.4 \pm 9.5\%$; $n=22$) than in patients with lower titers (mean $\pm$ SD $14.8 \pm 10\%$; $n=24$) ($P=0.013$), which is consistent with diminished autoantibody production in the presence of more efficient anergy.

DISCUSSION

We have developed and validated a simple flow cytometry–based assay that allows identification of ANA+ B cells by exploiting the same antigens used in current clinical tests. Using this assay, we confirmed the tolerance checkpoint limiting the entrance of autoreactive transitional B cells into the naive repertoire in both SLE patients and healthy subjects. However, we demonstrated an impairment of anergy induction in ANA+ naive B cells in SLE patients. Clinically, increased frequencies of the anergic ANA+ B cells were associated with decreased disease activity in our SLE cohort. Dysregulation of anergy in SLE has been suggested by a genetic study demonstrating that among 223 mouse cell types, T3 transitional B cells (which are thought to represent anergic naive B cells) had the expression profile most strongly associated with SLE risk loci (13,14). Although an expanded IgM^{low} population within the peripheral CD23+ B cell compartment of SLE patients has been reported, our gating strategy was different and excluded CD27+ B cells and transitional cells and focused only on naive B cells (CD19+CD27–IgD+CD38^{intermediate}CD10^{low/-}) (15). Anergic naive B cells in autoimmune patients may be more poised to exit that state, because IgM^{low} anergic B cells in SLE patients display an enhanced activation phenotype, expressing higher levels of CD95 and lower levels of CD22 (10). Indeed, abnormal up-regulation of the B cell activation markers CD80 and CD86 on IgM^{low} CD23+ B cells in SLE patients is purportedly driven by long-term exposure to self antigen (15). Interestingly, a transient loss of anergic B cells containing high-affinity and polyreactive insulin-binding B cells was observed in prediabetic patients and patients with new-onset type 1 diabetes (16).

There has been a recent interest in B cell-directed therapies, and BAFF blockade with belimumab has demonstrated modest clinical efficacy, specifically in patients with the anti-DNA serologic marker of SLE. An earlier longitudinal study of B cells in a subset of belimumab-treated patients showed that inhibiting BAFF selectively reduced the number of total transitional and naive B cells relative to pretreatment by 80% but had no effect on conventional memory B cells and plasma cells; the numbers of IgM+ memory cells and plasma cells were modestly reduced after prolonged treatment (17). These observations suggest that belimumab acts in patients in a manner similar to BAFF blockade in mice (12). Overexpression of BAFF in mice results in rescue of autoreactive B cells from tolerance; inhibiting BAFF limits the maturation of autoreactive transitional B cells, which have an increased requirement for BAFF (18–21). Our current analysis of B cell populations in SLE patients suggests that belimumab treatment may restore the censoring of ANA+ B cells by anergy. Importantly, a clinical trial of belimumab following B cell depletion is ongoing to determine whether it will lead to a less autoreactive reconstituted B cell repertoire.

We identified a tolerance checkpoint limiting the exit of autoreactive naive ANA+ cells into the CD27+IgD+ memory compartment in healthy controls and SLE patients (Figure 2). Despite extensive investigation, the nature and origin of CD27+IgD+ memory cells continue to be debated. Some investigators propose that CD27+IgD+ and CD27+IgD–IgM+ (IgM only) cells belong to a homogeneous subset deriving from germinal center (GC) reactions, with the latter small subset residing longer in the GC, acquiring more somatic mutation and down-regulating IgD (22,23). Other data suggest that the majority of the CD27+IgD+ memory compartment represents the circulating counterpart of splenic marginal zone B cells and develop outside of typical GC T cell-dependent interactions, and that IgM-only cells represent a distinct GC-derived memory subset (24–27). In any case, in SLE patients this memory subset represented a significantly smaller percent of the B cell compartment than it did in healthy controls. A similar reduction of this subset in SLE patients was recently reported and was correlated with higher levels of serum ANAs (28). Due to an activated phenotype and enhanced chemotactic activity, it was proposed that these cells were being recruited out of the bloodstream into lymphoid tissue in SLE patients, where they would presumably differentiate into switched memory or plasma cells (28). Marginal zone B cells in mice are enriched for autoreactivity and are associated with the production of autoantibodies in certain mouse models of lupus including the BAFF-overexpressing mouse, in which they are an expanded subset in the spleen (29–31). These data and the fact that most SLE patients have high BAFF levels would support the possibility that CD27+IgD+ cells are the human counterpart of murine marginal zone B cells and have a role in pathogenesis.

Although our data demonstrate more ANA+ B cells among CD27+IgD+ memory B cells in healthy controls than previously reported for IgM+ memory B cells (32), we observed reduced autoreactivity in this subset compared with naive B cells. We cannot fully explain the discrepancy, except to note that frequencies of ANA+ B cells in this subset were variable in our study, that the technologies used were different, and that we studied many more subjects. Our approach was influenced by B cell receptor density on the cell membrane, whereas the previous study relied on immunoglobulin messenger RNA quantities in the cell and the ease of obtaining both heavy chain and light chain transcripts by PCR. Furthermore,

CD27+IgD+ B cells trafficking in and out of the circulation may contribute to variability among subjects and reflect current antigen exposure; this hypothesis may explain the low frequency of antibacterial polyreactive B cells found in this compartment in the previous study.

A clear tolerance checkpoint for ANA+ B cells was observed between the naive and CD27+IgD– memory cell populations. The relatively low percentage of ANA+ B cells that we observed in the CD27+IgD– memory cell compartment contrasts with the high percentage observed in both SLE patients and healthy subjects in earlier studies (33,34). In the study by Mietzner et al, no differences were observed between SLE patients and healthy controls, and much of the autoreactivity arose via somatic mutation (34). As discussed above, discrepancies may be explained by the different technologies used and/or the number of and variability among individual subjects. Using a similar flow cytometry–based assay, we previously showed significantly reduced frequencies of DNA-reactive B cells entering the memory B cell pool from the naive B cell compartment in SLE patients but increased numbers of DNA-reactive memory B cells in association with active disease (35). Our data imply that cells other than classic CD27+IgD– memory cells (perhaps marginal zone, double-negative, and/or activated naive B cells) may be important sources of autoantibodies in SLE.

Double-negative cells have characteristics of memory cells, but their lineage and function are not clear. A proportion of double-negative cells contain an unusually long IgH third complementarity-determining region, a feature associated with autoreactive antibodies (24). We previously observed a subset of CD95+ double-negative cells to be correlated with disease activity in SLE (36). Interestingly, “activated” naive B cells have been identified in SLE patients as a unique subset generating a substantial proportion of ASCs (37). Thus, it is conceivable that activated naive B cells represent activated anergic B cells. Further support for the generation of autoreactive antibodies through nonconventional memory B cells or an extrafollicular pathway is the detection of germline-encoded autoreactive ASCs in SLE patients (37). A diagram of peripheral B cell relationships and tolerance checkpoints is shown in Supplementary Figure 5 (available on the Arthritis & Rheumatology web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.39710/abstract>).

In summary, we have developed an assay to phenotype ANA+ B cells and have shown a defect in anergy induction in autoreactive naive B cells that was restored in SLE patients treated with belimumab. This new assay enables prompt and inexpensive quantification of ANA+ B cells and easily accommodates large numbers of clinical samples. In addition to providing basic information on B cell biology and disease pathogenesis, direct measurement of such cells is likely to be useful in monitoring therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Identification of B cells that recognize nuclear antigens (ANA+ B cells) by flow cytometry, and validation of the assay. **A**, Composition of HeLa nuclear extracts analyzed by agarose gel electrophoresis (left), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (middle, left), and immunoblotting (right). **B**, Gating strategy for flow cytometry–based antinuclear antibody (ANA) assay, along with strategy used to sort single ANA+ and ANA–naive and transitional B cells for cloning of immunoglobulin genes. After gating on lymphocytes and excluding doublets by FSC and SSC (plots not shown), lymphocytes were further resolved into non-B cell (CD19–CD3+CD14+CD16+) and B cell (CD19+CD3–CD14–CD16–) populations. ANA+ B cells were identified using non-B cells as a negative control. **C**, Representative immunofluorescence staining patterns, on HEp-2–coated slides, of purified recombinant antibodies derived from sorted ANA+ (**a–e**) and ANA– (**f**) B cells from healthy controls, showing a variety of staining patterns: homogeneous (**a**), speckled (**b**), rim (**c**), centromere (**d**), and nuclear/cytoplasmic (**e**). **D**, ANA binding of recombinant antibodies from sorted ANA+ ($n = 23$) and ANA– ($n = 17$) B cells as measured by ANA enzyme-linked immunosorbent assay. Each data point represents the mean absorbance value of triplicates for each antibody sample. The red triangle in the ANA– group represents the negative control antibody B1. The dotted line represents the cutoff OD for ANA reactivity. Experiments were performed at least twice.

Figure 2.

Antinuclear reactivity in peripheral B cell subsets from systemic lupus erythematosus (SLE) patients and healthy control (HC) subjects. **A**, The gating strategy used to identify different B cell subsets. After resolving lymphocytes into non-B cells and B cells (as described in Figure 1B), CD27 and IgD staining was used to further define B cells into CD27+IgD+ memory, CD27+IgD- memory, double-negative (DN), and non-memory (CD27- IgD+) subsets. CD10 and CD38 expression was used to distinguish transitional (CD38^{high}CD10^{high}) and naive (CD38^{intermediate}CD10^{low/-}) B cells in the non-memory B cell subset. **B**, Zebra plots for a representative SLE patient, a healthy control subject, and a belimumab-treated SLE patient. B cells recognizing nuclear antigens (ANA+ B cells) were identified within each B cell subset. Non-B cells were used as a negative control to set the antinuclear antibody (ANA) gate. **C**, Scatterplots depicting the frequency of ANA+ B cells within each B cell subset in SLE patients (n = 37), healthy controls (n = 33), and belimumab-treated SLE patients (n = 9). Each data point represents the frequency in an individual subject; horizontal lines show the mean. P values were determined by 2-tailed Mann-Whitney test. NS = not significant.

Figure 3.

Frequency of anergic ANA+ naive B cells in SLE patients and healthy control subjects. **A**, Scatterplots depicting the proportions of ANA+ B cells that are transitional and naive in SLE patients (n = 37), healthy controls (n = 33), and belimumab-treated SLE patients (n = 9). Total ANA+ B cells were first identified as described in Figure 1B and subsequently gated on the B cell subsets described in Figure 2. **B**, Representative contour plots depicting IgM^{low} cells within the ANA+ naive B cell compartment of an SLE patient, a healthy control, and a belimumab-treated SLE patient. The gate for IgM^{low} B cells was determined individually for each sample, using non-B cells as a guide. **C**, Percentages of IgM^{low} B cells in the ANA+ naive B cell compartments of patients with SLE, healthy controls, and belimumab-treated SLE patients. P values were determined by 2-tailed Mann-Whitney test. In **A** and **C**, each data point represents the frequency in an individual subject; horizontal lines show the mean. Lo = low (see Figure 2 for other definitions).

Table 1

Specificity and sensitivity of the flow cytometry–based ANA assay*

Subject	Total no. of Antibodies Cloned	No. of true-positives (IFA+ Sort+)	No. of false-positives (IFA– Sort+)	No. of true-negatives (IFA– Sort–)	No. of false-negatives (IFA+ Sort–)	Specificity	Sensitivity
HC-3	25	10	2	10	3	10/12 (83)	10/13 (77)
HC-7	23	9	2	10	2	10/12 (83)	9/11 (81)
HC-8	45	22	4	16	3	16/20 (80)	22/25 (88)
SLE	37	15	3	15	4	15/18 (83)	15/19 (79)
Total	130	56	11	51	12	51/62 (82)	56/68 (82)

* Values for specificity are the no. of true negatives/no. of true negatives plus false positives (%). Values for sensitivity are the no. of true positives/no. of true positives plus false negatives (%). ANA = antinuclear antibody; IFA = immunofluorescence assay; Sort = sorted; HC = healthy control; SLE = systemic lupus erythematosus.

Table 2

Characteristics of the SLE patients and healthy controls*

Characteristic	SLE patients (n = 37)	Belimumab-treated SLE patients (n = 9)	P [†]	Healthy controls (n = 33)
Age, mean ± SD years	36.8 ± 12.7	45.9 ± 5.4	0.002	45.0 ± 18.0
Female	35 (94.6)	8 (88.9)	0.488	33 (100)
Ethnicity			0.156	
African American	22 (59.9)	3 (33.3)	–	1 (3.0)
Hispanic	5 (13.5)	4 (44.4)	–	1 (3.0)
White	7 (18.9)	2 (22.2)	–	27 (81.8)
Asian	3 (8.1)	0	–	3 (9.1)
Unknown	0	0	–	1 (3.0)
Disease duration, mean ± SD years	10.0 ± 6.5	15.3 ± 4.5	0.025	–
Current treatment				
Prednisone	24 (64.9)	2 (22.2)	0.029	–
Prednisone dose, mean ± SD mg	9.1 ± 14.2	1.4 ± 3.3	0.005	–
DMARD [‡]	21 (56.8)	1 (11.1)	0.023	–
Hydroxychloroquine	30 (81.1)	5 (55.6)	0.186	–
Current SLEDAI, mean ± SD	3.0 ± 2.7	1.1 ± 2.7	0.073	–
High anti-dsDNA antibody titer [§]	20 (54.1)	2 (22.2)	0.139	–
Hypocomplementemia	12 (32.4)	1 (11.1)	0.410	–
Anergic ANA+ B cells, mean ± SD %	9.6 ± 9.7	18.2 ± 10.8	0.024	14.7 ± 8.2

* Except where indicated otherwise, values are the number (%). SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; ANA + B cell = B cell recognizing nuclear antigens.

[†] SLE patients versus belimumab-treated SLE patients.

[‡] Disease-modifying antirheumatic drugs (DMARDs) include mycophenolate mofetil (n = 11), azathioprine (n = 8), methotrexate (n = 1), cyclophosphamide (n = 1), and tacrolimus (n = 1).

[§] Most anti-double-stranded DNA (anti-dsDNA) titers were determined by personnel at the Blood Testing Center, Northwell Health. Titers > 30 were considered positive, and titers < 12 were not recorded.

Table 3

Frequencies of ANA + B cells in SLE patients and healthy controls *

ANA+ B cells within B cell subsets	SLE patients (n =37)	Belimumab-treated SLE patients (n =9)	Controls (n =33)
Transitional	25.8 ±13.0	24.8 ±14.0	31.1 ±8.4
Naïve	10.0 ±5.1	16.4 ±10.0	13.1 ±5.8
CD27+IgD+	7.5 ±3.4	8.2 ±5.4	9.8 ±5.7
CD27+IgD–	1.4 ±1.1	1.6 ±1.6	2.7 ±2.1
Double-negative	3.6 ±2.7	4.4 ±4.5	5.1 ±3.5
IgM ^{low} naïve	15.6 ±8.4	19.0 ±11.1	21.8 ±9.5
IgM ^{intermediate/high} naïve	9.7 ±5.0	15.8 ±9.6	12.4 ±5.6
IgM ^{low} cells within ANA+ naïve subset	9.5 ±9.7	18.2 ±10.8	14.7 ±8.2

* Values are the mean ± SD. ANA + B cells = B cells recognizing nuclear antigens.