Diversity, cellular origin and autoreactivity of antibody-secreting cell population expansions in acute systemic lupus erythematosus

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Acute systemic lupus erythematosus (SLE) courses with surges of antibody-secreting cells (ASCs) whose origin, diversity and contribution to serum autoantibodies remain unknown. Here, deep sequencing, proteomic profiling of autoantibodies and single-cell analysis demonstrated highly diversified ASCs punctuated by clones expressing the variable heavy-chain region V_{H}4-34 that produced dominant serum autoantibodies. A fraction of ASC clones contained autoantibodies without mutation, a finding consistent with differentiation outside the germinal centers. A substantial ASC segment was derived from a distinct subset of newly activated naive cells of considerable clonality that persisted in the circulation for several months. Thus, selection of SLE autoreactivities occurred during polyclonal activation, with prolonged recruitment of recently activated naive B cells. Our findings shed light on the pathogenesis of SLE, help explain the benefit of agents that target B cells and should facilitate the design of future therapies.

RESULTS
Polyclonality of circulating ASCs during SLE flares
We obtained peripheral blood ASCs from patients with SLE who were experiencing disease flares while on minimal immunosuppression (Supplementary Table 1). Consistent with published observations3, ASCs defined as CD19^{+}IgD^{−}CD27^{hi}CD38^{hi} were up to 40-fold more abundant in these patients than in healthy subjects (Fig. 1a).
and included Ki67+CD138− and Ki67+CD138+ subsets (Fig. 1b). Thus, all circulating ASCs in SLE represented proliferative plasma blasts in various stages of maturation.

We sorted both of those ASC subsets, as well as naive cells (CD19+IgD+CD27+) and IgD− memory cells (CD19+IgD−CD27+), including isotype-switched and immunoglobulin M (IgM)-only cells, and sequenced their rearranged genes encoding antibody heavy chains. We first analyzed NGS data for clonal diversity. As in published studies of vaccination against influenza virus⁶ and multiple sclerosis⁷,⁸, we assigned sequences to individual clones (clonotypes) if they shared rearrangements of VH and joining heavy-chain (JH) segments, an identical length of immunoglobulin-heavy-chain complementarity-determining region 3 (HCDR3) and a Hamming identity of >85% for HCDR3 (Supplementary Note 1). Increasing the HCDR3 similarity to >90% did not substantially alter clonal assignments (data not shown). Therefore, we chose to use a threshold of >85% for the Hamming identity to allow a frequency of HCDR3 mutation in ASCs relative to that in their naive precursors similar to the mutation frequency present in HCDR1 and HCDR2 expressed by ASCs in published studies of responses to influenza virus⁶ and in our own data set (Supplementary Note 1 and Supplementary Figs. 1–3).

Given the scarcity of data available on the normative distribution of clonal size at steady state and during active immune response,⁶⁹ adjudication of abnormal clonotype expansion is necessarily arbitrary. Accordingly, we used several metrics to consistently measure clonal size, the contribution of large clones and repertoire diversity. The metrics assessed included the contribution by any clone to the total number of sequences (Fig. 1c) and the number of the largest clonotypes that accounted for 20% (D20) or 50% (D50) of all sequences (Supplementary Table 1). In addition, we classified many of the largest clones as having expanded substantially (Fig. 1d). In this context, ‘expanded lineages’ is a heuristic for the number of unusually large clones within a population, defined as the number of size-ordered lineages after the first occurrence of a change (‘delta’) of 0.1% between adjacent clone sizes. We analyzed samples before and after the removal of redundant sequences and observed a minimal change in clonal size relative to total size (data not shown).

ASCs from patients with SLE expressed a very polyclonal repertoire in which abnormally expanded clones represented a much smaller fraction of the total compartment and were substantially smaller than those from healthy control subjects either vaccinated with tetanus toxin or vaccinated against influenza virus (Fig. 1c,d, Supplementary Fig. 4 and Supplementary Table 1). Thus, the average D20 values for patients with SLE were 199.2 and 126.8 for CD138− ASCs and CD138+ ASCs, respectively, and 21.1 and 10.9, respectively, for the combined vaccinated groups (Supplementary Table 1). The corresponding average D20 values for patients with SLE were 1,721.8 and 1,181.6 for CD138− ASCs and CD138+ ASCs, respectively, and 159 and 125.9, respectively, for combined vaccination samples (Supplementary Table 1). The frequency of sequences that made up expanded CD138+ ASC clones, defined as meeting a difference threshold of 0.1% larger the previous clone in the size-ranked clones, was much lower in patients with SLE than in vaccinated healthy control subjects (Fig. 1c,d and Supplementary Table 1). Nonetheless, ASC populations from patients with SLE included a small number of sizable clonal expansions (2–10 and 0–18 expanded clonotypes in CD138− ASCs and CD138+ ASCs, respectively; Fig. 1d). This pattern was most prominent in patients SLE-2 and SLE-4 (Fig. 1d). Thus, the D20 values for CD138+ ASCs from patients SLE-2 and SLE-4 were only 26 and 31, respectively, with the largest clone in SLE-4 accounting for over 6% of 5 × 10⁴ unique sequences obtained from 2 × 10⁵ cells (Fig. 1d). In patient SLE-2, the largest clone accounted for 2.1% and 1.5% of all sequences identified from CD138− ASCs and CD138+ ASCs, respectively (Fig. 1d).

Naïve B cells from patients with SLE and vaccinated healthy control subjects were highly polyclonal (average D20 values: 473.2 for SLE, 554.5 for influenza virus, and 423 for tetanus toxin; Supplementary Table 1). However, significant clonal expansions (D20 = 3) were also
Figure 2. Isotype distribution and SHM in patients with SLE and healthy vaccinated control subjects. (a) Proportion of switched sequences (IgG + IgA) and unswitched sequences (IgM) in four cell subsets (above plots) from samples from patients with SLE (n = 5) and healthy subjects vaccinated against influenza virus (n = 4) or with tetanus toxoid (n = 4). IgD− memory cells include both isotype-switched cells and cells expressing IgM only. P < 0.05, IgM memory (t-test with unequal variances). (b) Heat-map histograms of mutation frequency (bottom half) for four populations of cells with three isotypes from 13 subjects (as in a); each column in a bar represents a single isotype and population from one subject; rows are histogram ‘bins’ indicating the frequency of mutations in V regions (bin size, 1%), with shades of gray (key) corresponding to bin height (beige, 0), divided by total sequences for the corresponding isotype, population and subject for normalization of heights (thus, bin heights sum to 1). Cyan lines indicate average of medians for each sample group. P < 0.05, ASCs from patients with SLE versus those from healthy vaccinated subjects (t-test unequal variances). Red bars (top) indicate frequency of sequences with a mutation rate below 3% with a significantly larger number of these sequences with low mutation rate compared with patients with SLE (SLE: 11.8% of sequences from CD138− ASCs in samples from SLE), than in healthy subjects (P < 0.05 (t-test with unequal variances)). (c) Replacement mutations (median frequency) in various regions of VH transcripts (above plots) in IgD− memory cells (top) and CD138+ or CD138− ASCs (bottom). Data are from thirteen independent experiments with one subject each (n values in a).

Present in the total naive compartment of patient SLE-2, possibly due to the presence of an activated fraction of cells in the total naive compartment (Supplementary Table 1 and Supplementary Fig. 5). This patient, who was on minimal immunosuppression, experienced a major acute flare accompanied nephritis and central nervous system, hematological and serological manifestations. Similarly, memory cells were highly polyclonal in the group vaccinated against influenza virus and in the group with SLE, but striking clonal expansions were displayed after vaccination of healthy control subjects with tetanus toxin (Supplementary Table 1 and Supplementary Fig. 5).

Clonal sizes calculated from sequence numbers could be influenced by mRNA abundance per cell and PCR-induced skewing. However, mRNA abundance should be similar within purified cell subpopulations. A notable exception could apply to populations that might contain a fraction of highly activated B cells with possibly higher expression of mRNA from the immunoglobulin heavy-chain locus (IGH). In this case, the estimated clonal size would be immunologically informative by identifying such population. Finally, we assumed that PCR skewing for any given VH segment should affect all subpopulations and clinical samples equally.

As expected, IgG and IgA isotypes represented the majority of sequences in ASCs (Fig. 2a). IgM provided a sizable contribution (5.42–19.53%) of all sequences in ASCs from patients with SLE, with substantial inter-subject variability in the contribution from IgM observed among vaccinated healthy control subjects (Fig. 2a). Notably, the frequency of IgM sequences in the IgD−CD27+ memory cell population was significantly greater for patients with SLE than for healthy control subjects after vaccination (20.9–68.1% and 1.5–37.5%, respectively; Fig. 2a). These numbers were consistent with the frequency of IgM+ memory cells observed by flow cytometry in 120 patients with SLE (median of 9.1%, with an upper limit of 60.6%; data not shown). In summary, ASCs from patients with SLE undergoing flares were highly polyclonal and contained, in addition to class-switched sequences, a substantial fraction of IgM sequences that presumably were derived from recently activated naive cells, as has also been seen after repeated vaccination with the human immunodeficiency virus gp120 vaccine10 and/or in IgM+ memory cells11.

Lower frequency of somatic hypermutation in SLE ASCs

We calculated the frequency of somatic hypermutation (SHM) by determining the length of the corresponding germline VH region in each sequence (approximately 285 base pairs (bp), although this varies by VH segment) and ascertaining the frequency of mutations in non-gap bases within that VH segment. ASCs from patients with SLE displayed a lower average mutation rate (4.98%) than that of ASCs from vaccinated healthy control subjects (7.33%) (Fig. 2b). Notably, samples from patients with SLE contained a large fraction of cells with less mutation, with 32.5% of sequences from CD138− ASCs and 30.8% of sequences from CD138+ ASCs containing fewer than 3% mutations (8.55 mutations per sequence, based on a germline VH segment of 285 bp), compared with 11.8% of sequences from CD138− ASCs and 10.0% of sequences from CD138+ ASCs in samples from vaccinated healthy control subjects (Fig. 2b). Patients SLE-4 and SLE-5 had a particularly large number of sequences in ASCs with less mutation (39.8 and 52.9%, respectively) (Fig. 2b). We confirmed these results by single-cell analysis of 771 isotype-switched ASCs...
obtained from six additional patients with SLE. In this cell population, 4% of the V_H sequences were not mutated, and sequences with less mutation averaged 18.5%, with a range of 7.1–38.5% among individual patients (data not shown).

The median mutation frequency was substantially higher in the hypervariable CDR than in framework regions (FRs) (an average of 6.74% (CDR) and 3.15% (FR), for patients with SLE, and 12.35% (CDR) and 4.57% (FR), for vaccinated healthy control subjects; Fig. 2c). The ratio of replacement mutations to silent mutations was also higher in the CDR: 5.83 replacement versus 0.00 silent (CDR), and 1.49 replacement versus 0.70 silent (FR), for patients with SLE; consistent with reference values for influenza virus–selected ASCs⁵ and the results obtained for healthy control subjects after vaccination (9.52 replacement versus 0.42 silent (CDR), and 2.92 replacement versus 1.49 silent (FR)). Thus, the pattern obtained for patients with SLE was consistent with antigen-driven selection of the precursors of ASCs, as expected for pre-existing memory cells and newly recruited naive cells driven through stimulation via the B cell antigen receptor (BCR).

SLE ASCs are punctuated by complex V_H4-34⁺ clonal expansion

We analyzed the use of IGH V_H segments to understand repertoire complexity. Of the 42–46 functional germline V_H segments available, V_H4-34 accounted for 5.9–19.5% of all sequences in ASCs from patients with SLE, but it accounted for a significantly lower fraction in vaccine responses (1.1–7.6%) and 1.6–11.1% for vaccination against influenza virus and the tetanus toxoid vaccine, respectively; Fig. 3a). In the top 20% of sequences (the D₂₀ fraction), this contrast was even more apparent, with CD138⁺ ASCs from patients with SLE containing an average of 20.7% V_H4-34 sequences, compared with 2.9% in the samples from healthy control subjects after vaccination. Similarly, V_H4-34 contributed an average of 19.8% of the D₂₀ fraction of sequences in CD138⁺ ASCs from patients with SLE but only 5.3% in the samples from healthy control subjects after vaccination. This highly autoreactive V_H segment has been found to correlate with active SLE in serological studies⁶,⁷, and in our studies it accounted for 21% of the largest ten ASC clones in patients with SLE and contributed substantially to clonal expansion in all five patients. In contrast, V_H4-34 contributed to ASC population expansion in only two of eight vaccinated healthy control subjects. Conversely, other V_H segments commonly expressed in the human antibody repertoire (V_H3-23 and V_H1-69) and overexpressed during infection with influenza virus, human immunodeficiency virus or hepatitis C virus (V_H1-69)¹² contributed significantly to ASCs from healthy control subjects responding to vaccination but much less prominently to ASCs from patients with SLE. Moreover, V_H4-34 was not common in ASC populations expanded during the active phase of other antibody-mediated autoimmune
diseases, including pemphigus vulgaris and IgG4-related disease. Thus, these findings ruled out the possibility of technical skewing and, in keeping with the high specificity for SLE of 9G4 antibodies, demonstrated ‘preferential’ population expansion of $\text{V}_{H4}\text{-}34^{+}$ ASCs from patients with SLE undergoing flares.

**SLE flares course with generalized ASC population expansion**

We analyzed samples from a larger number of patients with SLE by enzyme-linked immunospot (ELISPOT) assay to determine autoreactive and anti-microbial responses. We assessed autoreactivity to common SLE antigens, including dsDNA, Ro and Smith. We also assessed $\text{V}_{H4}\text{-}34$-encoded 9G4 antibodies, which contribute substantially to dsDNA responses and capture additional SLE autoreactivity, including antibodies to apoptotic cells and, thereby, provide a more encompassing measure of lupus autoreactivity. Despite the absence of recent immunization or likely natural exposure, cells specific for influenza virus or tetanus toxoid were much more frequent among ASCs from patients with SLE (range, 0–0.3% or 0–0.6%, respectively, of all IgG$^{+}$ ASCs) than their undetectable frequency among ASCs from healthy control subjects (Fig. 3b). In contrast, even in patients with large expansions of ASC populations, anti-dsDNA, anti-Ro and anti-Smith responses common to SLE together accounted for less than 3% of IgG-producing ASCs (Fig. 3c). Of note, these frequencies closely matched similar autoimmune responses in a mouse model of lupus.

The median frequency of 9G4$^{+}$ ASCs was 2.4% and, consistent with published results, could account for up to 20% of all IgG$^{+}$ ASCs. Thus, circulating ASCs from patients with SLE had enhanced responses to infectious antigens as well as autoantibodies typical of SLE. The latter, however, accounted for only a limited fraction of all ASCs and, in keeping with the NGS data, were dominated by 9G4 antibodies.

**Contribution of naive cells to ASCs in SLE flares**

ASCs originate either from pre-existing memory B cells or from newly acN cells through germinal center (GC) or extrafollicular reactions, with both pathways having a prominent role in mouse models of lupus. To determine the contribution of naive cells to ASC population expansion in SLE, we analyzed the NGS data for connectivity using the 85% clonal-identification-metric method (Supplementary Note 1). We quantified results using a correction score to account for the variability introduced by differences in the number of input cells in separate subjects as well as sequences available. Patients with SLE displayed a significantly higher degree of naive cell–to–ASC connectivity than did vaccinated control subjects (Fig. 4a,b). Thus, the average ratio of the connections between IgD$^{-}$ memory cells and ASCs to the connections between naive cells and ASCs was significantly lower in SLE than in recall responses elicited by the vaccination of healthy subjects (Fig. 4c). Notably, naive B cells in the patients with the highest frequency of ASCs with less mutation (SLE-4 and SLE-5)

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### Figure 4

**Figure 4** Contribution of naive cells to ASC populations in SLE flares. (a) Molecular features of the antibody repertoire in four sorted B cell and ASC populations (outer perimeter) from patient SLE-4 and subject FLU-3 (day 7 after vaccination against influenza virus), presented as circular plots (Circos software); numbers along perimeter indicate sequence identification number; within that, clonal size (segmentation of rings); middle, connections between populations (only between naive cells and ASCs, and/or between IgD$^{-}$ memory cells and ASCs); for ASCs, color indicates lineages accounting for the D$_{20}$ segment (sequences found in the largest clones up to 20% of total sequences), and grey sections indicate sequences found in the other 80% of sequences (all sequences of naive cells and IgD$^{-}$ memory cells were highly polyclonal (gray)). (b) Molecular features of the antibody repertoire for patient SLE-4, limited to $\text{V}_{H4}\text{-}34$ sequences (presented as in a). (c) Relatedness of ASCs to naive cells and to IgD$^{-}$ memory cells, presented the ratio of the fraction of all IgD$^{-}$ as memory clonotypes connected to ASCs to the fraction of all naive lineages connected to ASCs (l$\text{L}_{\text{Naiv}}$ + ASC/l$\text{L}_{\text{Naiv}}$ + ASC), where ‘L’ indicates linkage, ‘SwM’ indicates switched memory, and ‘Na’ indicates naive (top), and frequency of ASC lineages with a median mutation frequency of <3% (bottom). (d) Alignment of individual sequences in the $\text{V}_{H4}$ segment from the largest clone identified in patient SLE-4 against those in naive cells, CD138$^{-}$ASCs and CD138$^{+}$ ASCs. Data are representative of nine independent experiments (a; n = 5 samples (patients with SLE experiencing an acute flare) and n = 4 samples (healthy subjects vaccinated against influenza virus)) or five independent experiments (b; n = 5 samples (patients with SLE experiencing an acute flare)) or are from thirteen independent experiments (c; n = 4–5 samples per condition).
Figure 5 Characterization of acN cells in SLE. (a) Fractionation of CD19*IgD*CD27− cells from a healthy, non-vaccinated subject (left), a patient with SLE without flare (middle) and a patient with SLE during an acute flare (right), by CD24 expression and staining with MTG (full data set, e). Numbers adjacent to outlined areas indicate percent CD24pos−negMTG+ (resting) cells (blue), CD24*MTG+ (transitional) cells (green) or CD24−MTG* (activated) cells (red). (b) Flow cytometry of acN cells (IgD*CD27−MTG*CD24+) and resting naive B cells (IgD*CD27*MTG*CD24−) from a patient with SLE experiencing an acute flare, assessing various markers (below plots). (c) Flow cytometry of B cells from an SLE patient experiencing an acute flare, identifying the CD19hi and CD19hi fractions (top), and analyzing the CD24 expression and MTG staining of those fractions (bottom) (full data set, d). Numbers above bracketed lines indicate percent CD19+ cells (left) or CD19hi cells (right) (top), or 9G4− cells (left) and 9G4+ cells (right) in those fractions (bottom). (d) Frequency of autoreactive 9G4+ B cells among resting CD19hi cells and the activated CD19hi fraction of IgD*CD27− cells (presented as in Fig. 3b). (e) Frequency of MTG−CD24+ cells (left), MTG+CD24+ to CD24hi cells (middle) or MTG*CD24− cells (right) in samples from healthy control subjects and patients with SLE (green ‘x’ indicates active flare) (presented as in Fig. 3b). (f) Frequency of CD23− cells among IgD*CD27− naive cells (activated cells) in the naive compartment of patients with SLE analyzed longitudinally during periods that included moderate and severe flares in the context of persistent disease activity. *P < 0.0005 and **P < 0.00005 (t-test, unequal variance). Data are representative of 30 experiments with one subject each (a,e; n = 6 samples (healthy control subjects), n = 9 samples (patients with SLE without flare), and n = 15 samples (patients with SLE experiencing an acute flare)), 21 experiments with one subject each (b; n = 6 samples (healthy control subjects) and n = 15 samples (patients with SLE experiencing an acute flare)), 15 experiments with one subject each (c,d; n = 15 samples (patients with SLE experiencing an acute flare)), or 13 experiments (f; n = 13 samples (patients with SLE)).

acN cells as precursors of ASCs in SLE

The simultaneous presence of clonally related naive cells and ASCs suggested persistent activation and differentiation of naive B cells. Thus, we analyzed the phenotype and antibody repertoire of circulating naive B cells during SLE flares. Multi-chronic flow cytometry has demonstrated that CD19+IgD*CD27− cells, commonly classified as mature naive B cells, include three distinct populations defined by staining with MitoTracker Green (MTG), a mitochondrial dye retained by transitional and activated cells but not by resting naive cells, and by expression of the cell surface marker CD24. In healthy control subjects, the CD19+IgD*CD27− population was overwhelmingly dominated by resting naive B cells (Fig. 5). Patients with SLE had substantial expansion of the MTG*CD24pos−hi transitional cell population (Fig. 5), as has been reported in experiments using alternative markers. In addition, flaring SLE was also characterized by large increases in MTG*CD24− cell populations that were nearly absent from healthy control subjects (Fig. 5a,e). This population was characterized by a CD19hiCD21−CD38loIgMloCD23− phenotype (Fig. 5c). While an IgD*MTG+ phenotype is also shared by late transitional cells, the lack of expression of CD24 and CD38, low expression of IgM and the absence of CD10 mitigated against this alternative. Instead, we surmise that this subset represented recently acN cells. In support of this interpretation, activated B cells show concomitant upregulation of CD24 expression and downregulation of CD21 expression and/or retention of MTG. This contention was also supported by concordance with disease activity during longitudinal analysis (Fig. 5f). Of relevance, CD19hi activated B cell populations showed considerable enrichment for autoreactive 9G4+ B cells (Fig. 5c), a finding consistent with the prominent contribution of V(19)4−34+ naive cells to the clonal expansion of ASCs.
Figure 6 Clonality and connectivity of acN cells in SLE. (a) NGS data (presented as in Fig. 3a) showing the clonality of acN cells from four patients with SLE, with two analyzed at multiple time points (t1–t5 over 8 weeks (SLE-3) and t1–t4 over 4 weeks (SLE-6)); orange bars along right margins indicate lineages connected to ASCs; blue bars at top indicate frequency of acN lineages connected to ASCs that were identified in top 50% of sequences from size-ranked clones (D50). (b) Clonal expansion and clonal lineage connectivity of acN cells (IgD+CD27+MTG+CD24−) with ASC populations during an SLE flare at the third time point (t3) for patient SLE-3; inner ‘track’ indicates clonality (colored section; only for the top 50% of sequences); outer ‘track’ indicates mutation frequency for constituent sequences. (c) Alignment of a clone (purple dots (ASCs) and purple arrow (acN cells)) in b containing sequences present in acN cells, CD138+ ASCs and CD138− ASCs, against the corresponding germline sequence (VH4-59) and the CDR3 contained in the sequence with the fewest mutations in VH (common ancestor); red indicates mismatches relative to the reference sequences. (d) Alignment of amino acid sequences from acN cells with no or low mutation and ASCs with considerable mutation within a large VH4-34+ clone from patient SLE-6 at a single time point; red indicates mutations relative to the germline sequence. (e) Alignment of the nucleotide sequence of a single cell obtained from acN cells (top) and clonally related sequences derived from ASCs by NGS 4 months earlier at the time of flare in patient SLE-3. Data are from 11 independent experiments (a; n = 11 samples from four patients with SLE subjects) or one experiment with one subject (b–e).

From a repertoire standpoint (Fig. 6a), the salient properties of acN cells were increased clonality (D20 index 28.7-fold higher than that of naive cells from healthy control subjects) and a high degree of ASC connectivity, with up to 32.5% of all sequences from acN cells and nine of the ten largest acN cell clones representing clonal precursors of ASCs (Fig. 6a,b). Of note, these measurements varied considerably during longitudinal analyses. This suggested a highly dynamic population and indicated that, as it might be predicted given the necessary lag time between activation of naive cells and clonal expansion of their ASC progeny, the single-time-point analysis substantially underestimated the contribution of newly activated B cells (Fig. 6a).

Another distinctive feature of acN cells was a substantial frequency of SHM (an average of 2.37% in six samples, compared with only 0.95% for resting naive B cells; data not shown), a process associated with GC cells that can also occur in activated follicular precursors of ASC17,24. The rarity of mutation in resting naive cells indicated that this result could not be explained either by sequencing errors or V H polymorphisms.

To better understand the dynamics of expanded populations of ASCs in lupus and their naive precursors, we constructed their phylogenetic relationships with IgTree software25. This tool identified complex clones in which naive cells with no SHM or low degree of SHM (0–2%) co-existed with ASCs with a large ‘load’ of SHM (mutation frequency of up to 21.5%). As examples, we aligned sequences from large clonotypes that stemmed from an acN cell without mutation and included CD138+ ASCs and CD138− ASCs that were simultaneously present in the circulation (Fig. 6c,d). We were able to generate similar trees for 55% of the top 20 connected clones analyzed (data not shown). Given the rate of SHM in antigen-driven mouse autoimmune B cells26, these data would be best explained by prolonged persistence and diversification of acN cells in human SLE, as has been proposed in mouse models24. This model was supported by the ready identification at the single-cell level, in a patient with SLE, of acN B cells clonally related to mutated ASCs detected by NGS 4 months earlier (Fig. 6e). Combined, our results indicated that large naive B cell clones persisted and recirculated for several months in patients with SLE.

**Contribution of ASC clonal expansion to serum autoantibodies**

To confirm the NGS results and establish the functional consequence of ASC population expansion, we obtained affinity-purified serum 9G4 antibodies from one informative sample from a patient with
SLE experiencing an acute flare and characterized the antibodies by liquid chromatography–tandem mass spectrometry. We identified candidate antibodies by mapping high-confidence peptide spectra. We searched mass spectra in the SEQUEST data-analysis program used for protein identification against NGS databases obtained from distinct populations of B cells and ASCs isolated from the same blood sample. We established perfect identity between the full-length heavy-chain protein sequence and NGS sequences for 39 serum antibodies representing 20 distinct clonotypes. Two clonally related serum antibodies that shared the same HCDR3 peptide (Fig. 7a) were identical matches with two ASC sequences (Fig. 7b, c) in the largest VH4-34+ clone found in patient SLE-3. Suggestive of a derivation from naive cells, the protein and corresponding ASC nucleotide sequences were almost completely devoid of any mutation (Fig. 7b). Consistent with prolonged persistence of ASCs and ongoing antibody production, both the serum 9G4 antibody clonotype and the corresponding ASC clones persisted in the circulation for over 8 weeks (Supplementary Fig. 6). Thus, persistent ASC clones directly derived from naive cells contributed substantially to the serum autoantibody repertoire in SLE.

**Single-cell analysis of ASC autoreactivity and origin**

The autoreactivity of 9G4 monoclonal antibodies derived from naive and memory cells from patients with SLE has been documented.
To establish the applicability of those findings to ASCs, we sorted 9G4+ plasmablasts from a patient with SLE who had been analyzed before the single-cell analysis, as well as clonally related sequences (VK1-39) from ASCs (bottom). Data are from one experiment (n = 9 monoclonal antibodies (a) or n = 1 monoclonal antibody (b)).

**DISCUSSION**

Understanding the provenance, repertoire and serological consequences of ASC population expansion is essential for delineating the pathogenesis of SLE and for therapeutic insights. We found that these ASCs expressed a highly polyclonal repertoire punctuated by large clonal expansions dominated by autoreactive VH4-34+ cells and shaped the serum autoantibody compartment. This profile was present even in a patient with very acute SLE with major multi-organ involvement.

Our study of SLE has delineated the connectivity among B cell subsets during an ongoing autoimmune response. Indeed, a relevant observation was that the two major subsets of circulating ASCs (CD138− ASCs and the more mature CD138+ ASCs) were highly interconnected and represented newly produced proliferative plasmablasts in the process of ongoing maturation.

Polyclonal ASCs could indicate activation of bystander B cells or antigen-driven stimulation by multiple lupus autoantigens. Serological studies of human SLE have argued for generalized activation, whereas in mice, antigenic drive has been favored.

The NGS data and simultaneous increases in anti-microbial and autoreactive IgG autoantibodies from germline sequences without the need for SHM or affinity maturation.

**Figure 8** Autoreactivity of 9G4+ ASCs in SLE. (a) Autoreactivity of monoclonal antibodies (mAb) (left margin, clone number and CDR3 sequence) from single cells among 9G4+ ASCs sorted from patient SLE-3 at 4 months after an SLE flare, as well as switched memory cells (SwM) (negative control), assessed against ANA (by Hep-2 immunofluorescence (ANA IFA) and enzyme-linked immunosorbent assay (Hep-2 ELISA)) and dsDNA, and as the binding of apoptotic cells (ApopCB) and B cells (BCB), measured as described, and as reactivity to chromatin, Ro, cardiolipin, ribosomal P antigens (Fig. 8a), or influenza virus (Flu) and tetanus toxin. (b) Immunofluorescence microscopy analyzing the autoreactivity of monoclonal antibody 652-F6 (from a) to various doses (above images) of ANA, showing nuclear and cytoplasmic staining of cells. Original magnification, ×40. (c) Alignment of sequences in the VH4-34 region to those of clone 652-F6 (from a) and sequences of a large clonal tree identified by NGS of bulk ASCs 4 months before the single-cell analysis, as well as clonally related sequences (VK1-39) from ASCs (bottom). Data are from one experiment (n = 9 monoclonal antibodies (a) or n = 1 monoclonal antibody (b)).
ASCs in our study would favor the former possibility. Nevertheless, these mechanisms are not mutually exclusive, since multiple cytokines and stimulation via Toll-like receptors can induce activation of bystander cells while promoting clonal expansion of autoreactive cells receiving BCR-transduced signal 1 (refs. 33,34). Indeed, that last component is supported by the clonal expansion of VH4-34 ASCs with disease-associated autoreactivity.

A central finding of our study was that during SLE flares, a substantial contribution to ASC expansion was derived from acN cells that survived for months while still serving as precursors of dynamically diversifying ASCs. Of note, our analysis must have underestimated the participation of naive cells, as the expansion of naive precursor cell populations would occur days to weeks before the generation of ASCs. Moreover, our stringent criteria would have low sensitivity for the detection of clonal relationships between naive cells and mutated ASCs. The relevance of acN cells was emphasized by their temporal relationship with active disease; the high clonality index, suggestive of antigen-driven population expansion; enrichment for autoreactive 9G4+ B cells; and the high frequency of IgD-IgM+ memory cells, the first memory ‘layer’ generated from naive cells in both a GC-dependent fashion and a GC-independent fashion1. Enhanced participation by naive cells would also help explain the lower levels of SHM displayed by ASCs from patients with SLE relative to that of memory cells.

Our results provide new insight into the phenotype, magnitude and downstream consequences of the activation of naive B cells in SLE. Indeed, the previously recognized population expansion of CD21+ activated B cells was ascribed to CD27+B cells, while other studies did not discriminate among transitional and/or recent immigrant and mature naive B cell subsets, a limitation overcome by incorporation of the analysis of MTG staining and CD24 expression. Also, in contrast with published studies, in our experiments, acN cells were CD23−, a finding consistent with the downregulation of CD23 expression induced by sustained activation of the BCR, Toll-like receptor 9, and interferon-α and interferon-γ, all important stimulatory pathways in SLE. Population expansion of ASCs during lupus flares might be expected to represent recall responses due to chronic exposure of patients to autoantigens, as has been shown for multiple sclerosis. So how do acN cells in patients with SLE compete with memory cells presumed to have higher responsiveness, affinity and precursor frequency? Such properties remain to be assessed in chronic human autoimmunity, particularly in SLE, in which many variables and serum factors may enhance the competitiveness of naive B cells without mutation. Published studies have demonstrated substantial polyreactivity of naive B cells to nonspecific antigens, including dsDNA, chromatin and ribosomal P antigens. We also demonstrated that acN B cells were directly selected into the autoimmune effector compartment without the need for affinity maturation. This feature is consistent with extrafollicular generation of lupus autoantibodies, a pathway that, while well defined in mouse models, has remained unexplored in human SLE. The importance of our findings is enhanced by the demonstration of accelerated lupus-like autoimmunity mediated by GC-independent IgM autoantibodies.

Our study indicated very long persistence of peripheral naive B cells and ASCs in SLE. Mature peripheral B cells in rodents have a lifespan of 2–6 weeks, a time frame shortened to just a few days for autoreactive naive B cells. The only in vivo human study available has estimated the half-life of circulating naive cells at 22 d (ref. 48). We found that naive cells without mutation were present concurrently with clonally related ASCs with mutational loads expected to accumulate over many weeks, according to estimates made for mice of the lpr (lymphoproliferation) strain. Moreover, we were able to readily identify at the single-cell level autoreactive acN cells without mutation 4 months after initial detection by NGS. Such prolonged survival could be explained, at least in part, by the high concentrations of B cell–activation factor characteristic of SLE. While normal plasmablasts undergo apoptosis within a few days, the persistence of ASC clones in the circulation for at least 8 weeks would suggest a much longer lifespan in SLE. This is consistent with the prolonged survival of plasmablasts reported in autoimmune mice deficient in the tyrosine kinase Lyn.

In summary, we have demonstrated active SLE characterized by large polyclonal expansions of newly generated ASC populations. These ASC populations, however, also contained substantial clonal expansions of antigen-specific autoreactive cells, of which a sizeable fraction was derived from persistently acN cell and, at least in some cases, without the need for SHM. The aggregate of our results is consistent with a model of sustained and asymmetric differentiation of acN cells through both extrafollicular pathways and GC reactions; a possibility that best explains the composition of genealogical clonal trees observed at a single time point in the blood.

Our study provides an immunological explanation for the clinical benefit of belimumab (antibody to B cell–activation factor) and epratuzumab (anti-CD22), which ‘preferentially’ affect acN cells. The B cell populations described here represent candidate biomarkers for the analysis of patient disease activity, prediction of flares and design of personalized therapies. At a fundamental level, the study of clonally expanded acN cells and their highly diversified progeny should help elucidate the nature of the potentially distinct antigens that trigger the initial activation and subsequent selection of autoreactive B cells in SLE.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. SRA: NGS data, SRP057017.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.M.T. obtained most samples, conducted sample preparation and cell sorting, designed and conducted the NGS studies and analyzed and interpreted the data, helped to design figures, and helped to write the manuscript; C.F.E. and A.F.R. wrote the programs used for NGS analysis, helped in data interpretation, produced visualization of the data, and helped to design and produce the figures; J.D., I.G., S.S. and W.C.C. conducted and analyzed the proteomics studies; A.C. conducted the experiments with single-cell monoclonal antibodies; T.I. conducted the ELISPOT experiments; J.H. obtained samples from patients with pemphigus, conducted some sequencing studies and helped with the analysis; S.J. helped with sequencing analysis; R.F.P. provided samples from patients with pemphigus; R.M. provided the IgTree program and aided in analysis; C.W. provided flow cytometry data and helped with its analysis; F.E.-H.L. provided the samples from vaccinated subjects and aided in data analysis; and I.S. designed and supervised the project, helped in experimental design and analysis, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Subjects. Four subjects vaccinated with trivalent vaccine against influenza virus, four subjects vaccinated with tetanus vaccine, and eight patients with SLE experiencing acute flares were enrolled in this study at the University of Rochester Medical Center and Emory University between 2010 and 2013. All studies were approved by the Institutional Review Boards at the University of Rochester Medical Center and Emory University School of Medicine, and informed consent was provided by all study subjects. Healthy subjects received the vaccination against influenza virus or tetanus toxoid (the tetanus-diphtheria (Td) vaccine or the combination tetanus-diphtheria-pertussis (Tdap) vaccine) as part of routine medical care. The recruitment of patients with SLE not during the annual influenza season and patient history were used to determine absence of recent immunization or likely natural exposure to influenza. Recent tetanus vaccination was also ruled out by the patient history provided. Peripheral blood mononuclear cells were isolated before vaccination and on days 6–9 after vaccination for all vaccinated subjects; two subjects with SLE who were experiencing active flares were monitored longitudinally at weekly time points for up to 8 weeks. Patients with SLE fulfilled four or more criteria of the modified American College of Rheumatology classification (http://www.rheumatology.org/Practice/Clinical/Indexes/Systemic_Lupus_Erythematosus_Disease_Activity_Index_SELENA-Modification/) and were routinely evaluated by expert rheumatologists at the University of Rochester Rheumatology Clinic and the Emory Lupus Clinic. Patients were recruited if classified as having a moderate-to-severe flare according to the flare index of Safety of Estrogen in Lupus: National Assessment–Systemic Lupus Erythematosus Disease Activity Index and were on minimal immunosuppression at the time of flare (only hydroxychloroquine and/or <10 mg prednisone or equivalent glucocorticoid per day of).

Multi-color flow cytometry and sorting. Mononuclear cells were isolated from peripheral blood by ficoll density-gradient centrifugation and were stained with the following antibody staining reagents directed against human molecules: fluorescein isothiocyanate (FITC)–conjugated goat F(ab′)2 anti–human IgD (IA6-2), phycoerythrin (PE)–iododicarbocyanine (Cy5)–conjugated goat F(ab′)2 anti–human IgM (G02-127), Pacific Blue–conjugated anti-CD38 (HB7), PE-Cy7–conjugated anti-CD23 (M-L233), PE-Cy5–conjugated anti-CD21 (B-ly4), PE-conjugated anti-CD27 (L128) and allophycocyanin–Pacific Orange–conjugated anti-CD14 (TK4), Pacific Orange–conjugated anti-CD3 (UCHT1), PE–Alexa Fluor 610–conjugated anti-CD29 (G04-29), PE–Alexa Fluor 610–conjugated anti-CD21 (B-ly4), PE–Alexa Fluor 610–conjugated anti-CD20 (L243) and allophycocyanin–Pacific Orange–conjugated anti-CD123 (8F11), Pacific Orange–conjugated anti-CD14 (TK4), Pacific Orange–conjugated anti-CD19 (L28), and Pacific Orange–conjugated anti-CD27 (L128) and PE–Alexa Fluor 610–conjugated anti-CD29 (G04-29), and MitoTracker Green. Approximately 0.1 × 10⁵ to 3 × 10⁵ cells were collected for each population with a FACSAria II (BD Biosciences).

NGS of the IGH repertoire. Total cellular RNA was isolated (cell quantity, Supplementary Table 1) with an RNeasy Micro kit by following the manufacturer’s protocol (Qiagen). Approximately 2 ng of RNA was subjected to reverse transcription with an iScript cDNA synthesis kit (BioRad). Aliquots of the resulting single-stranded cDNA products were mixed with primers specific for Vµ1-Vµ7 framework region 1 (50 nM) and primers specific for constant regions Cµ, Cγ, and Cδ (250 nM) preceded by the respective Illumina Nextera sequencing tag (oligonucleotide sequences listed below) in a PCR volume of 25 μl (4 μl template cDNA) with High Fidelity Platinum PCR Supermix (Invitrogen). Amplification was performed with a Bio-Rad C1000 Thermal Cycler (Bio-Rad) with the following conditions: after an initial step of 95 °C for 5 min, 25–40 cycles (depending on amplification efficiency) of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, ending with a final extension step of 72 °C for 5 min. Products were purified with RapidTips2 (Diﬃnity Genomics), and Nextera indices were added via PCR with the following conditions: 72 °C for 3 min, 98 °C for 30 s, 5 cycles of 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 3 min. Ampure XP beads (Beckman Coulter Genomics) were used for purification of the products, which were subsequently pooled and denatured. Single-strand products were subsequently sequenced on a MiSeq (Illumina) with the 500 cycle v2 or 600 cycle v3 kit. Primer sequences were as follows: Vµ1a, 5′-CAGGATCAGCTTGCTGAC-3′; Vµ1b, 5′-SAGGTCCAGCTTGATAC-3′; Vµ1c, 5′-CARATGCCAGCTGTTGCG-3′; Vµ2, 5′-CAGGCTCACATTGAGG-3′; Vµ3, 5′-GGTCCCTGAGACTCTCGT-3′; Vµ4, 5′-ACCCCTGTCCCTACCTGC-3′; Vµ5, 5′-GCCAGCTCGTGACCTCG-3′; Vµ6, 5′-CAAGACTGCTAGAACCCCTG-3′; Vµ7, 5′-CAGGCTACGGTGGCAA-3′; Cµ3, 5′-CAGGAGACGGGAGGGA-3′; Cγ1, 5′-CCGATGGCCCTGGTGGA-3′; Cγ2, 5′-GAAGACCTGGGCTGGTGGA-3′, F tag, 5′-TCGTCGCGAGCCTGACTGTTAAAGAGACGAC-3′, and R tag, 5′-GTTCCTCGTGCGCTGGAGATGGTGTATAGGACAGAC-3′.

Bioinformatics analysis of NGS data. An informatics pipeline developed in-house was used for analysis of sequencing data (full methodology for processing, visualization and analysis of data by this pipeline, Supplementary Fig. 1). After paired-end reads were joined, sequences were filtered on the basis of a length and quality threshold. Sequences less than 200 bp and sequences with poor overlaps (>8% difference in linked region) and/or a large number of bp below a threshold score (sequences containing more than 15 bp with less than a quality value (Q) of Q30, 10 bp with less than Q20, or any bp with less than Q10 scores) were excluded from further analysis. Isotypes were then determined by analysis of the constant-region segment of each sequence, and a random subset of 150,000 sequences were aligned and analyzed for cladology and for mutations in the V region with data provided by the IMGT/HighV-QUEST web portal (from The International Immunogenetics Information System). The subset of 150,000 sequences was necessary for the limitations imposed by IMGT/HighV-QUEST v1.3.1 at the time of preparation of this manuscript. The subset of sequences was also used to relieve computational stress and allow analysis in reasonable timing. Samples tested in subsets of multiple sizes did not result in any substantial difference in the cladology or our interpretation of results. After alignment, the sequences were analyzed by a custom program written by the authors in perl and matlab (Supplementary Data Set 1). Sequences were filtered by removal of ‘unproductive’ and ‘unknown’ sequences, and clusters of sequences were identified on the basis of the clonal identification metric (described below). 50,000 sequences from the final set were then randomly chosen, again to relieve computational stress of the downstream analyses, for retention of similar number of sequences in each data set and for display purposes. All data from IMGT/HighV-QUEST were retained through the process and were used for mutation calculations and alignment analyses. The frequency and distribution of somatic hypermutation were ascertained on the basis of non-gap mismatches of expressed sequences with the closest germline Vµ1 sequence. Mutation frequencies were determined by calculation of the number of mutations in V regions relative to the number of bases in non-gap V regions. The ratio of replacement mutations to silent mutations were calculated for CDR and framework regions through use of the average of the median mutation frequencies by IMGT/HighV-QUEST in the corresponding Vµ1 areas. Circular visualization plots were created with Circos software v0.64. Clonally expanded genes and primer validity were confirmed by 5′ rapid amplification of cDNA ends of cells obtained from patients with SLE and sorted as single cells, as well as cells from healthy vaccinated control subjects (two replicates were analyzed, and 97% of the products from ASCs were also found in the PCR-based assay; all expanded clones were identified). Validation of the quantification of Vµ14-34 sequences was verified by naive cells from healthy control subjects ‘spiked’ with the Ramos human cell line, which expresses a rearranged Vµ14-34 region (0%, 25%, 50% and 75% ‘spike-ins’ resulted in 0%, 29.54%, 44.48% and 79.6% RAMOS-specific sequences, respectively).

Phylogenetic analysis. Phylogenetic analysis was used to determine clonal relationships between different B cell subsets and to analyze the structure and diversification of B cell clones. Clonal sequences were aligned to germline sequence with Clustal X software, and genealogical relationships were constructed with the IgTree program, which uses a distance method–based algorithm to find the most likely tree with the highest probability23. This software allows for the possibility that not all intermediate sequences within a B cell clone may have been identified.

Antigen-specific ELISPOT assay. Antigen-specific enzyme-linked ELISPOT assays were performed as described1. Peripheral blood mononuclear cells or sorted ASCs or B cell subsets were added to 96-well ELISPOT plates (96 wells; MAIPPS510) coated with anti–human IgG (5 μg/ml; A80-304A; Bethyl),
anti–human IgA (5 µg/ml; 62-7400; Invitrogen), Trivalent Influenza Vaccine 2009-2010, 2010-2011 (6 µg/ml; Sanofi Pasteur), or monoclonal antibody 9G4 (provided by F.K. Stevenson). After overnight incubation, wells were washed, and bound antibodies were detected with alkaline phosphatase–conjugated antibody to human IgG; (109-055-008), to human IgA (109-055-011) or to human IgM (109-055-129) (all from Jackson Immunoresearch; all used at a concentration of 1 µg/ml) and results were developed with Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories). Spots in each well were counted with a CTL immunospot reader (Cellular Technologies). Results are represented as the ratio of antigen-specific spots to total IgG spots.

**Serum IgG proteomics.** Serum proteomics was used for analysis of serum antibodies by nano-flow liquid chromatography coupled to tandem mass spectrometry as described. The 9G4+ antibody fraction was purified from the serum of two of the patients with SLE by affinity chromatography with monoclonal antibody 9G4. 9G4+ fractions were eluted and their expression of 9G4 was verified, followed by digestion with chymotrypsin, pepsin, elastase or trypsin and analysis by liquid chromatography–tandem mass spectrometry. High-confidence peptide spectral matches of V regions were obtained with SEQUEST against a reference database created by next-generation DNA sequencing of the various B cell subsets from the same patient with the same blood sample.

**Recombinant single-cell monoclonal antibodies.** Recombinant single-cell monoclonal antibodies were generated and their autoreactivity was analyzed as described by nested RT-PCR analysis of RNA isolated from single cells sorted from the populations of interest into 96-well plates.

**Enzyme-linked immunosorbent assay (ELISA).** QUANTA Lite ELISA of ANA, dsDNA, chromatin and ribosomal P by (INOV A Diagnostics) and Total Anti-Cardiolipin Screen ELISA (ALPCO Diagnostics) were performed according to the manufacturer’s instructions as described.

**ANA immunofluorescence.** Slides coated with Hep-2 (Nova lite Hep-2; 708101; INOV A Diagnostics) were incubated for 30 min in a moist chamber at ambient temperature with 20 µl purified antibodies at a concentration of 30 µg/ml, then were washed in PBS, and incubated for 30 min with FITC-labeled goat antibody to human IgG (20 µl; 508123; INOA Diagnostics). ANA-negative control serum and ANA-positive control serum was included in all experiments. Samples were examined on a confocal microscope (Olympus FV1000/TIRF; Emory University Integrated Cellular Imaging Microscopy Core). Positive staining was determined by comparison with the control samples assessed at equal exposure times.

**Statistical analysis.** Statistical comparisons between groups were performed with a two-sample t-test with unequal variances. In some cases involving comparisons of lineage counts or cases in which we did not assume normally distributed data, we used a Wilcoxon rank sum test. All statistical tests were computed with Matlab (The Mathworks).