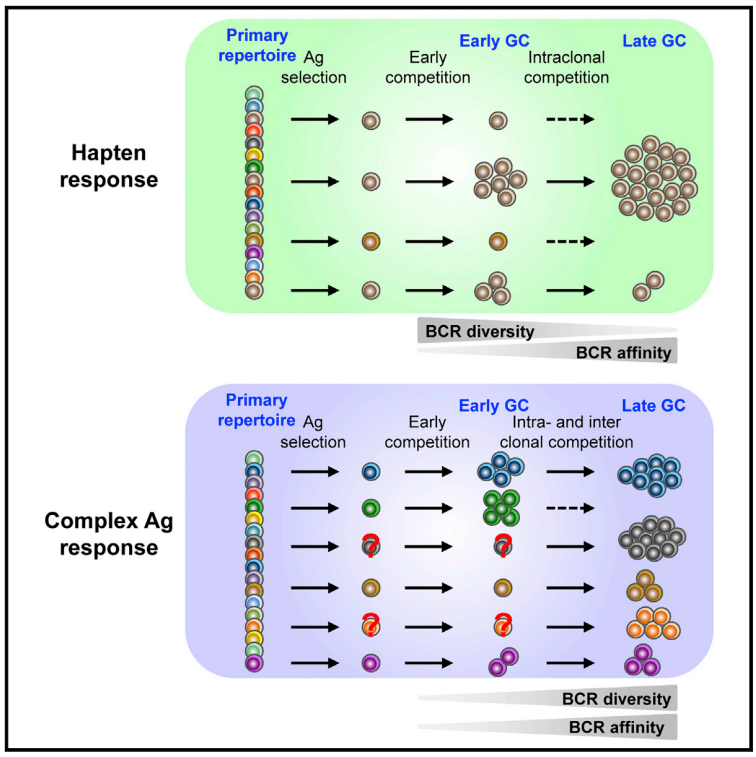
Article

Immunity

Complex Antigens Drive Permissive Clonal Selection in Germinal Centers

Graphical Abstract



Authors

Masayuki Kuraoka, Aaron G. Schmidt, Takuya Nojima, ..., Stephen C. Harrison, Thomas B. Kepler, Garnett Kelsoe

Correspondence

ghkelsoe@duke.edu

In Brief

B cell selection and affinity maturation in germinal centers (GCs) elicited by complex antigens are not well studied. Kelsoe and colleagues demonstrate that affinity maturation in GCs is accompanied by increased clonal diversity and large variances in inter- and intraclonal BCR avidities in response to anthrax protective antigen and influenza hemagglutinin.

Highlights

- Developed a single B cell culture to characterize the mouse B cell repertoire
- Affinity maturation of GC B cells is accompanied by increased V_H representation
- GCs permit a wide range of intra- and interclonal BCR affinities
- Substantial fractions of GC B cells do not detectably bind native antigen

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Complex Antigens Drive Permissive Clonal Selection in Germinal Centers

Masayuki Kuraoka,[1] Aaron G. Schmidt,[3] Takuya Nojima,[1] Feng Feng,[4] Akiko Watanabe,[1] Daisuke Kitamura,[5] Stephen C. Harrison,[3,6] Thomas B. Kepler,[4,7] and Garnett Kelsoe[1,2,*]
[1]Department of Immunology
[2]Human Vaccine Institute
Duke University, Durham, NC 27710, USA
[3]Laboratory of Molecular Medicine, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
[4]Department of Microbiology, Boston University School of Medicine, Boston, MA 02118, USA
[5]Research Institute for Biomedical Sciences, Tokyo University of Science, Noda, Chiba 278-0022, Japan
[6]Howard Hughes Medical Institute, Boston, MA 02115, USA
[7]Department of Mathematics and Statistics, Boston University, Boston, MA 02118, USA
*Correspondence: ghkelsoe@duke.edu
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SUMMARY

Germinal center (GC) B cells evolve toward increased affinity by a Darwinian process that has been studied primarily in genetically restricted, hapten-specific responses. We explored the population dynamics of genetically diverse GC responses to two complex antigens—*Bacillus anthracis* protective antigen and influenza hemagglutinin—in which B cells competed both intra- and interclonally for distinct epitopes. Preferred V_H rearrangements among antigen-binding, naive B cells were similarly abundant in early GCs but, unlike responses to haptens, clonal diversity increased in GC B cells as early "winners" were replaced by rarer, high-affinity clones. Despite affinity maturation, inter- and intraclonal avidities varied greatly, and half of GC B cells did not bind the immunogen but nonetheless exhibited biased V_H use, V(D)J mutation, and clonal expansion comparable to antigen-binding cells. GC reactions to complex antigens permit a range of specificities and affinities, with potential advantages for broad protection.

INTRODUCTION

Our knowledge of clonal selection in humoral responses comes largely from studies of genetically restricted antibody (Ab) responses to haptens, most notably 2-phenyl oxazolone (Berek et al., 1991), *p*-azophenylarsonate (Manser et al., 1984), and (4-hydroxy-3-nitrophenyl)acetyl (NP) (Jacob et al., 1991). These haptens have been studied because they elicit responses dominated by B cells that express genetically restricted B cell antigen receptors (BCRs) and are amenable to analysis. For example, immunogenic conjugates of NP elicit in *Igh*^{b} mice humoral responses dominated (>90%) by B cells expressing V(D)J rearrangements comprising the V_H1-72 and Vλ1 gene segments (Bothwell et al., 1981; Jacob et al., 1991).

Somatic hypermutation (SHM), clonal selection, and affinity maturation take place in germinal centers (GCs) (Berek et al., 1991; Jacob et al., 1991, 1993; Takahashi et al., 1998). Characteristically, as the GC reaction to haptens progresses, the clonal diversity of GC B cells wanes and limited sets of somatically mutated, higher-affinity B cells prevail; late GC responses are characteristically dominated by descendants of a few ancestor cells (Jacob et al., 1993). In the case of anti-NP Abs, for example, affinity maturation results in the frequent recovery of B cells bearing the V_H1-72 gene segment with a particular V_H point mutation (W33L) from late GCs (Allen et al., 1988; Dal Porto et al., 1998; Weiss and Rajewsky, 1990).

Although experimentally tractable, genetically restricted humoral responses are atypical. Abs to complex protein antigens represent genetically diverse, polyclonal humoral responses driven by various epitopes arrayed across the antigen (Benjamin et al., 1984; Laver et al., 1990). B cells responding to these complex antigens are clonally distinct, and in GCs they compete both intra- and interclonally. That is, competition occurs within clonal lineages for a single epitope and between lineages recognizing distinct epitopes. Because interclonal competition plays at most a minor role in restricted Ab responses to haptens (Jacob et al., 1993), models for clonal selection in GCs have focused largely on affinity-driven competition for single epitopes (Berek et al., 1991; Dal Porto et al., 2002; Jacob et al., 1991, 1993; Shih et al., 2002).

Humoral immunity elicited by infection or vaccination reflects the dynamics of concomitant intra- and interclonal selection. A necessary first step toward understanding such responses is to characterize naive, mature B cells that bind antigen and to trace this population into and through the GC reaction. The technical challenge is to analyze the BCR somatic genetics (paired V(D)J and VJ rearrangements) and the phenotypes (specificity and avidity) of individual B cells.

To overcome some of the limitations of current methods for single B cell characterization (Wardemann et al., 2003; Wrammert et al., 2008), we developed a single B cell culture method that supported the proliferation and plasmacytic differentiation of mature and GC B cells. With this tool, we characterized antigen-driven selection and affinity maturation in polyclonal B cell

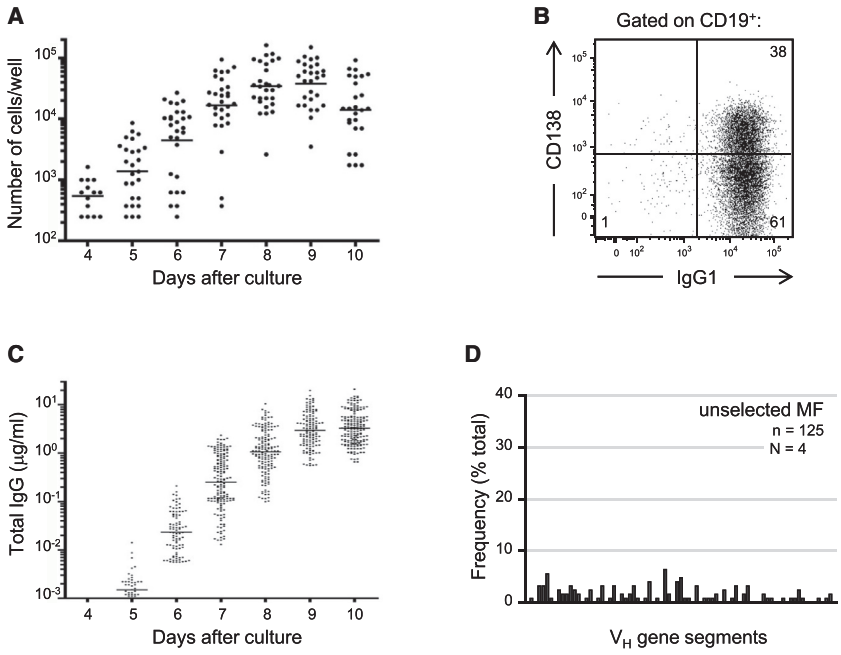

Figure 1. Single-Cell Cultures Support Proliferation, Class-Switch Recombination, and Ab Production of B Cells

Single MF B cells were sorted from the spleens of naive B6 mice and cultured with NB-21.2D9 feeder cells.

(A) Kinetics of increasing B cell numbers after single-cell culture. Live B cells were determined by Trypan blue exclusion and distinguished from NB-21.2D9 cells by size. Each symbol represents individual wells (n = 14–29). Bars indicate geometric mean.

(B) Representative flow diagram for surface IgG1 and CD138 expression by the progeny of single B cells after 9 days of culture.

(C) Kinetics of clonal IgG accumulation during the course of single-cell culture. Supernatants (n = 308) were harvested from individual wells on days 4 to 10, and IgG concentrations were determined by ELISA. Symbols represent individual wells and bars indicate the geometric mean of IgG^+ samples.

(D) V_H gene segment usage of unselected MF B cells (n = 125) isolated from naive B6 mice (n = 4). Data are representative of two or more independent experiments. See also Figure S1 and Tables S1–S3.

populations elicited by immunization with recombinant *Bacillus anthracis* protective antigen (rPA) or influenza hemagglutinin (rHA); our characterizations began with antigen-binding, mature naive B cells and followed clonal selection and affinity maturation through the GC reaction for up to 16 days. We found, as expected, that the frequencies and avidities of antigen-binding B cells increased significantly over the transition from pre-immune, naive B cells to late GC B cell populations. Affinity maturation of BCRs during GC responses was accompanied by accumulation of V(D)J mutations, but also by large variation among both inter- and intraclonal BCR avidities and by *increased* clonal diversity. The degree of variability of intraclonal BCR avidities appears to be at odds with models of affinity maturation by clonal competition (Dal Porto et al., 2002; Jacob et al., 1993; Schwickert et al., 2011; Shih et al., 2002), and increasing clonal diversity in GCs elicited by rPA and rHA contrasts with the purifying selection and oligoclonal GCs that characterize anti-hapten responses (Berek et al., 1991; Jacob et al., 1991, 1993). We propose that clonal selection in GCs is permissive for a wide range of BCR affinities and that lower-affinity GC B cells, and those less fit in other ways, might remain in GCs for substantially longer periods than generally thought.

RESULTS

Single B Cell Cultures Provide Representative Sample of BCR Repertoires

To establish efficient and non-selective cultures for single B cells (Nojima cultures), we introduced by retroviral transduction mouse IL-21 cDNA into the $CD154^+$ 40LB fibroblast cell line (Nojima et al., 2011), creating the NB-21 feeder cell line. We then screened a panel of 53 NB-21 transductants for their capacity to support B cell proliferation, plasmacytic differentiation, and immunoglobulin G (IgG) secretion. A single, optimized feeder clone, NB-21.2D9 (Figure S1A), was selected and used in all experiments. Individual, mature follicular (MF) B cells sorted onto NB-21 feeder layers proliferated extensively ($\geq$15 divisions) and reached a population plateau (mean = 3.7×10^4) after 9 days of culture (Figure 1A). By day 9, virtually all (>95%) progeny cells expressed surface IgG1, and about 40% differentiated into $CD138^+$ plasmablasts or plasmacytes (Figure 1B). IgG was first detected in culture supernatants at day 5 (~1.5 ng/mL); subsequently, IgG concentrations increased logarithmically until day 9 (2.8 [±0.3] μg/mL) (Figure 1C).

To ascertain whether the V(D)J diversity of these Nojima cultures accurately reflected the primary B cell repertoire, we amplified, cloned, and sequenced cDNA from V(D)J transcripts present in single-cell cultures (McWilliams et al., 2013; Rohatgi et al., 2008; Tiller et al., 2009). Analysis of V(D)J sequences from cultured B cells confirmed that virtually all (1,330/1,331) individual B cell cultures (Tables S1 and S3) contained a single, in-frame V(D)J rearrangement. Further, V_H and Vκ gene segment usage among unselected MF B cells over the *Igh* and *Igκ* loci (Figures 1D, S1E, Tables S1 and S2) was compatible with patterns of V_H/V_κ usage determined by other means (Aoki-Ota et al., 2012; Kantor et al., 1997). Frequencies of D and J segment usage and CDR3 lengths (Figures S1C–S1G) also mirrored prior reports (Aoki-Ota et al., 2012; Kantor et al., 1997), indicating that these cultures did not select for particular BCR types. Finally, all V_H gene segments recovered from unselected MF B cells were germline (data not shown).

To determine whether Nojima cultures also provided representative samples of the GC B cell repertoire, we characterized GC B cell populations in BALB/c mice immunized with hen ovalbumin conjugated with the NP hapten (NP-Ova) in alum (Table S3). The Ab response to the NP hapten (NP^a response) is characterized by nearly equal frequencies (53%:47%) of κ- and λ-bearing IgG Abs (White-Scharf and Imanishi-Kari, 1981) and

Table 1. ELISA Summary of Single B Cell Cultures

B Cell Source[a]	Control Mature	Ag-Selected	GC (Day 8)	GC (Day 16)
rPA Immunization				
IgG^+/total screened[b]	230/275	1,779/3,036	681/2,442	498/2,200
Cloning efficiency	84%	59%	28%	23%
IgG (μg/mL)[c]	3.8	2.9	1.0	1.1
rPA^+IgG^+/IgG^+[d]	0/230	45/1,779	214/681	275/498
rHA Immunization				
IgG^+/total screened[b]	244/308	2,704/5,181	1,136/3,080	731/3,080
Cloning efficiency	79%	52%	37%	24%
IgG (μg/mL)[c]	6.7	5.0	2.7	1.0
rHA^+IgG^+/IgG^+[d]	0/244	29/2,704	294/1,136	366/731

Mice were immunized with rPA or rHA in alum via footpad. Single B cells sorted from the draining LNs were expanded in single B cell cultures. Frequencies and concentrations of total and specific IgG in culture supernatants were determined by ELISA. See also Figure S2 and Table S4.

[a]Control mature B cells were sorted from either naive mice or immunized mice. Antigen (Ag)-selected, mature B cells were sorted from spleen of naive mice. GC B cells were sorted from immunized mice at indicated time points.

[b]Number of IgG-positive samples/number of samples screened.

[c]Average (geometric mean) IgG concentrations in individual culture supernatants.

[d]Number of Ag-binding IgG-positive samples/number of IgG-positive samples.

the λ^+ fraction of the NP^a response is significantly enriched for the V_H14-3 gene segment (Loh et al., 1983). From 113 Nojima cultures of single GC B cells that were positive for IgG, 83 (73%) bound NP; 59% of these carried the κ L-chain and 41% the λ L-chain (Table S3). In contrast, 90% of the IgGs that bound Ova or NP-Ova or that did not react with any component of the NP-Ova immunogen had κ L-chains (Table S3). In our survey, 30/33 (91%) of NP-binding, clonal IgG/λ Abs were encoded by V_H14-3 whereas none of 38 (0%) NP-specific IgG/κ Abs were (Table S3). Single naive or GC B cells cultured on NB-21.2D9 feeder cells efficiently proliferated and differentiated to IgG plasmblasts irrespective of BCR specificity and avidity; Nojima cultures provided representative samples of BCR diversity.

Robust GC Reactions after Immunization with rPA or rHA

To characterize the B cell populations that responded to rPA or rHA, we first established the kinetics of serum Ab responses and GC reactions after a single immunization in a mouse hind leg with antigen in alum precipitates (Figure S2). In general, serum IgG Abs were first detected on day 8 after immunization and rose continuously to reach concentrations of ~40 (rPA IgG) or ~5 (rHA IgG) μg/mL by day 20 (Figures S2A and S2B).

Immunization with rPA or rHA also elicited robust GC responses in the draining LN with similar magnitude and kinetics (Figures S2C and S2D). GC B cell ($B220^{hi}GL\text{-}7^+Fas^+IgD^-$) numbers first increased on days 4–6 after immunization, reached a peak at day 12, and precipitously dropped by day 16. GC B cell numbers then remained constant until day 20 (Figures S2C and S2D). The decrease in GC B cell numbers from days 12 to 16 was consistent with intensive selection; consequently, we chose day 8 and day 16 GC B cells to represent the BCR repertoires of early and late GC B cell populations, respectively.

Selection by rPA or rHA Increases the Frequencies of Specific B Cells 4,000-fold in GCs

To characterize the population structure and dynamics of antigen-specific B cells before (pre-immune) and after immunization (GC responses) with rPA or rHA, we sorted mature ($B220^+CD93^-IgL^+$) B cells that bound rPA- or rHA-fluorochrome conjugates from naive mice and we sorted GC B cells ($B220^+GL\text{-}7^+CD38^{lo}IgD^-CD93^-CD138^-$) from the draining LNs of immunized animals on day 8 (early) or day 16 (late) after immunization (Figures S2E and S2F). GC B cells were not sorted on the basis of specificity. Single, sorted B cells were cultured for 9–10 days with NB-21.2D9 feeders and culture supernatants containing clonal IgG were screened by ELISA for antigen binding.

We obtained 8,003 clonal IgG Ab examples from control MF B cells (n = 474), rPA- or rHA-binding MF B cells (n = 4,483), day 8 GC B cells (n = 1,817), and day 16 GC B cells (n = 1,229). The average concentrations of clonal IgG Abs ranged from 1.0 to 6.7 μg/mL (Table 1); no rPA- or rHA-binding IgG Abs were recovered from control MF B cells (0/474), whereas 2.5% (45/1,779) and 1.1% (29/2,704) of clonal IgG Abs from antigen-selected MF B cells were rPA or rHA specific, respectively (Table 1). Approximately 0.5% of the total MF B cell population was labeled by rPA or rHA (Figure S2F), so we estimated the frequencies of mature B cells that avidly bind rPA or rHA (sufficient to be detected as secreted IgG) to be 1.3×10^{-4} (1/8,000) and 5.6×10^{-5} (1/18,000), respectively.

Frequencies of antigen-binding, clonal IgG Abs in GC B cell populations were several orders of magnitude higher than the naive, mature B cell pool. Antigen-specific cells from early GCs constituted 26% (rHA, 294/1,136) to 31% (rPA, 214/681) of the cloned population, and in late GCs those frequencies rose to 50% (rHA, 366/731) and 55% (rPA, 275/498) (Table 1). To ensure that these binding frequencies represented binding to native epitopes rather than determinants generated by adsorption to ELISA plates, we inhibited a subset (n = 91) of HA-specific clonal IgGs with soluble rHA (2 μg/mL). ELISA titers for 88/91 (97%) of HA-specific IgGs were reduced by an average of 70%, whereas heterologous inhibition by rPA had no effect. Therefore, antigen-driven selection into the GC response increased the representation of specific B cells in late GCs ≥4,000-fold above their frequencies among naive, mature B cells.

Affinity-Dependent Selection of GC B Cells Is Complex

To determine the distribution of BCR affinities for rPA and rHA in MF and GC B cell populations, we determined an avidity index (AvIn) for each clonal IgG Ab that detectably bound rPA or rHA (Figure 2). This index represented the ratio of antigen-specific binding of clonal IgG Ab to that of a monoclonal IgG Ab standard specific for the appropriate antigen. The IgG standard used for rPA was BAP0105 (Abcam), an IgG1/κ that binds to both the 83 and 63 kDa forms of PA; the K_D of BAP0105 (70×10^{-9} M) was determined by surface plasmon resonance. The HA standard was a murinized (Cγ1/Cλ1) version of the human CH67 rAb (Schmidt et al., 2013; Whittle et al., 2011), which recognizes the receptor binding

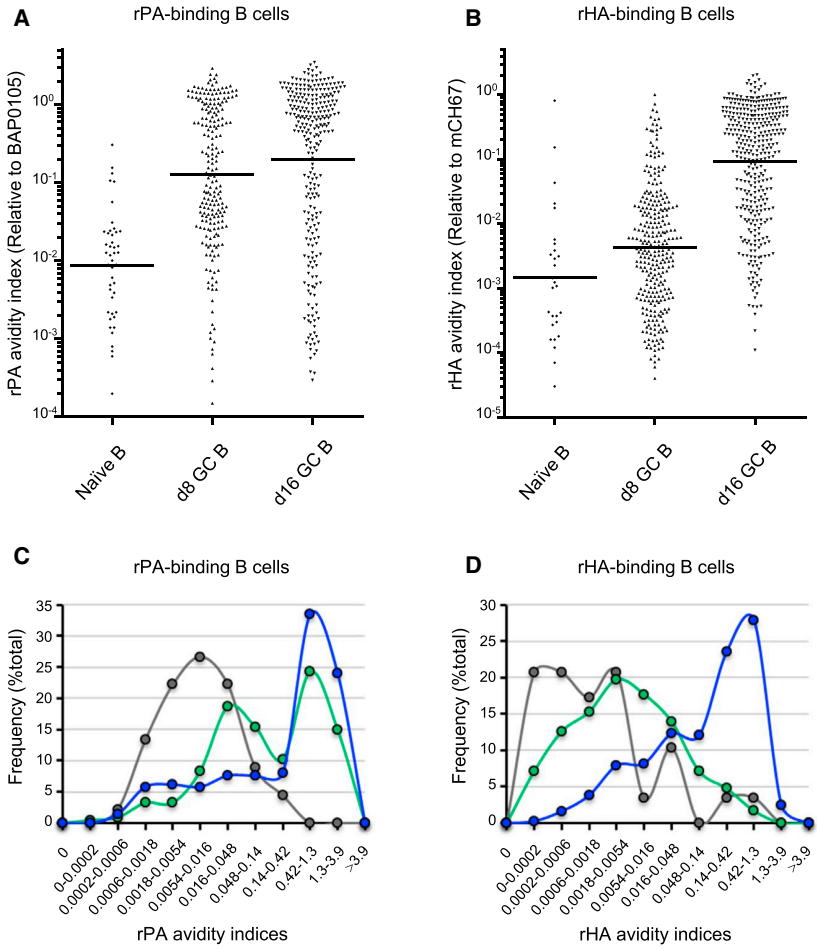

Figure 2. Affinity-Dependent Selection of GC B Cells Is Complex

Distributions of AvIn of culture supernatant IgGs from individual, single B cell cultures.

(A and B) Each symbol represents an AvIn value for an individual clonal IgG sample (n = 29–366). Bars indicate geometric means.

(C and D) Distributions of rPA and rHA AvIn for indicated B cell subsets (gray, selected, naive, mature B cells; green, day 8 GC B cells; and blue, day 16 GC B cells). Curves were created by binning with 3-fold intervals AvIn values for all samples in each B cell cohort.

Combined data from two or more individual mice are shown. See also Figures S3 and S4 and Table S4.

site of HA and neutralizes a variety of H1N1 influenza virus strains. The measured K_D of CH67 for rHA is 2.4×10^{-9} M.

To determine how AvIn correlate with measured K_D values, we generated a series of 14 rAbs by expressing V(D)J rearrangements recovered from single GC B cell cultures that secreted antigen-specific IgG; the K_D values of the resulting rPA (n = 6) and rHA (n = 8) IgG Abs were then measured and plotted against the AvIn determined from the culture supernatants (Figure S3). The AvIn values and measured K_D for both rPA (R^2 = 0.99; p = 0.017) and rHA (R^2 = 0.88; p = 0.016) binding Abs were strongly correlated and extend over a wide range of index values (Figure S3). We conclude that AvIn are robust and meaningful estimates of relative BCR avidity.

Median AvIn for rPA-binding clonal IgG increased ~12-fold from rPA^+ selected, pre-immune, MF B cells (AvIn = 0.012) to day 8 GC B cells (AvIn = 0.14; p = 0.0001) (Figure 2A). Continuing selection increased the median AvIn ~4-fold further in day 16 GC B cells (AvIn = 0.57; p = 0.003 versus day 8 GC AvIn) (Figure 2A). Median rHA AvIn also increased during humoral responses, with a relatively modest, 4-fold increase from rHA^+ selected pre-immune MF B cells (AvIn = 0.001) to early day 8 GCs (0.004; p = 0.009); this modest selection was followed by a 20-fold increase in avidity in day 16 GC B cells (0.18; p < 0.0001) (Figure 2B). Thus antigen selects in two distinct stages, first by biased recruitment/retention of naive B cells into the GC response (Schwickert et al., 2011) where clonal competition leads to additional rounds of selection for increasing BCR affinity (Jacob et al., 1993; Takahashi et al., 1998).

To analyze the distribution of BCR avidities for rPA and rHA within populations of cells, we binned AvIn values from individual samples in 3-fold intervals to generate histograms for BCR populations (Figures 2C and 2D). The BCR AvIn of rPA^+ selected, pre-immune, mature B cells had an approximately normal distribution around a median AvIn (0.0054–0.016), which was 0.54%–1.6% of the BAP0101 standard (Figure 2C). This distribution was consistent with unbiased selection for a set of randomly generated BCR paratopes. By day 8 after immunization, the distribution of rPA AvIn for GC B cells became bimodal, with one peak (0.016–0.048) at approximately three times the median for rPA-binding B cells from naive mice and a second (0.42–1.3) comparable to the BAP0105 Ab standard (Figure 2C); early GC responses included substantial populations of higher- and lower-avidity B cells. That the Abs included in the lower peak had substantially higher avidities than the median compartment of pre-immune population samples indicated avidity-dependent selection into the GCs and/or avidity-dependent selection in GC between days 5 and 8 (Figure 2C). With the progression of GC responses to day 16, the lower-avidity peak of early GC populations disappeared, leaving a single, higher AvIn peak and a long "tail" of lower-avidity cells (Figure 2C). About 70% of day 16 samples fell within the high-avidity peak.

Distributions of rHA AvIn in pre-immune, early, and late GC B cells followed similar patterns but with differences that might be significant (Figure 2D). Although the AvIn of most (~80%) rHA^+ selected, pre-immune, mature B cells clustered lower, at a median value 0.0004 (range: 0.00003–0.0054, an AvIn 0.03% to 0.54% of the CH67 standard), two samples had AvIn nearly equal to CH67 (Figure 2D). Unlike the bimodal distributions of AvIn for rPA-binding, early GC B cells, the distributions for rHA-binding early GC B cells were broader, with a peak at 0.0018–0.0054, 2- to 5-fold higher than the median rHA-binding

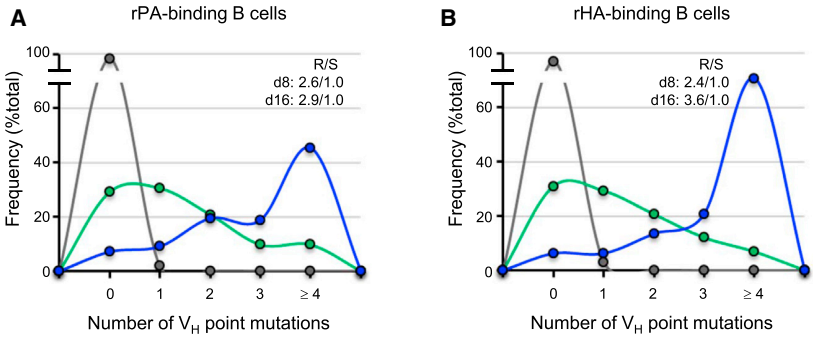

Figure 3. SHM in GC B Cells Alone Does Not Account for Affinity-Dependent Selection

Distribution of V_H point mutation numbers among rPA-binding (A) or rHA-binding (B), naive, mature B, and GC B cells. V_H gene segments were identified and V_H point mutations enumerated by comparison to germline. Gray, selected naive mature B cells (n = 44 and 26 for rPA-binding [A] and rHA-binding [B] B cells, respectively); green, day 8 GC B cells (n = 72 and 58); and blue, day 16 GC B cells (n = 154 and 113) isolated from two or more individual mice. Abbreviation is as follows: R/S, ratio of replacement/silent mutations. Combined data from two or more independent experiments are shown.

mature B cells (Figure 2D). The broad distributions of BCR avidities among day 8 GC B cells indicated that any affinity requirement to enter these GC reactions was not stringent (Dal Porto et al., 2002). By day 16, however, the distributions of rHA AvIn became similar to those of late rPA GC B cells, with a single peak at high AvIn values and an extensive tail of lower values (Figure 2D).

Although the GC responses elicited by rPA or rHA exhibited substantial affinity maturation, they also allowed the persistence of B cells with substantially lower-avidity BCRs (Figures 2C and 2D). To reconcile this apparent contradiction, we developed a simple mathematical model in which the probability of GC B cell division increased linearly with the cumulative distribution function on BCR avidity up to a threshold value beyond which cells divide with certainty (Figure S4). This simple and plausible selection model closely recapitulated the complex avidity distributions we observed for rPA and rHA GCs. Affinity-driven selection of GC B cells responding to protein antigens might have a significant stochastic component and the retention of GC B cells with lower-avidity BCRs might be advantageous for humoral protection.

SHM in GC B Cells Alone Does Not Account for Affinity Maturation to rPA and rHA

To examine the somatic genetics of antigen-driven selection of B cells, we amplified paired V(D)J and VJ rearrangements from cell pellets of individual IgG^+ cultures and subsequently cloned and sequenced amplicand products. We performed genetic analysis on subsets of clonal cultures: antigen-binding MF B cells in naive mice (n = 70, 95% of total), day 8 GC B cells (n = 130, 26%), and day 16 GC B cells (n = 267, 42%).

As expected (Kuraoka et al., 2009), we found no V_H mutations (1.1×10^{-4}; 2/18,550 base pairs sequenced) in antigen-binding, mature B cells (Figure 3), whereas V_H mutations in individual GC B cells became increasingly abundant over time. rPA and rHA GC responses had average mutation frequencies of 0.48–0.58×10^{-2} at day 8 and 1.2–1.4×10^{-2} at day 16, respectively. Approximately 70% and 90% of day 8 and day 16 GC B cells, respectively, carried V_H point mutations (Figure 3). As for hapten responses (Jacob et al., 1993), GC B cells accumulated V_H mutations; about half (47%) of day 16 GC B cells carried ≥ 4 V_H mutations whereas only 8% of day 8 GC B cells did (Figure 3). Broadly, V_H mutation frequencies were correlated with increasing AvIn but this general relationship did not mirror the bimodal and very broad AvIn distributions of early GCs (compare Figures 2 and 3). Mechanisms in addition to SHM, e.g., intrinsic BCR affinity and downregulation of BCR expression (George and Claflin, 1992), mediate B cell selection in early GCs.

Increasing Diversification of V_H Usage in GCs Induced by rPA and rHA

The distribution of V_H gene segment usage in unselected MF B cells appeared unbiased (Figure 1D), whereas recovery of select V_H gene rearrangements characterized both rPA and rHA responses. From rPA-binding, pre-immune, MF B cells and day 8 GC B cells, we recovered V_H8-8 and V_H1-82 rearrangements at high frequency (Figure 4A). These two V_H gene segments accounted for 50% (36/72) of recovered V_H gene segments in early GC but by day 16 the frequency of V_H1-82 gene segments fell to ~15% and V_H8-8 gene segments were more rare (~2%) (Figure 4A). Instead, B cells infrequently recovered from day 8 GCs, e.g., those with V_H1-26 rearrangements, appeared in late GCs (Figure 4A). V_H gene usage among rPA GC B cells at day 16 was more diverse than in day 8 GCs; the early dominance of select V_H gene segments in pre-immune and early GC B cells was not stable.

rHA-binding, pre-immune, MF B cells were more diverse but enriched for V(D)J rearrangements containing the V_H1-82, V_H1-81, V_H1-80, V_H1-72, or V_H1-7 gene segments (15/26; 58%). These same V_H gene segments were abundant in day 8 GCs (18/58; 31%) (Figure 4B). As for rPA GCs, V_H gene segments that dominated the early rHA responses became less frequent (16/113; 15%) in late GCs and B cells that were rare early in the response (e.g., V_H1-78, V_H1-69, or V_H14-2 rearrangements) grew more common (Figure 4B). This pattern of increasing clonal diversity was quite distinct from that of hapten-specific GCs (Jacob et al., 1993). Unlike responses to haptens, the early "winners" in GC responses to rPA and rHA were displaced by B cells that were initially rare but grew frequent in late GCs. This observation implies that fitness within the GC is not explained by initial BCR avidity or predominance in the pre-immune repertoire and early GCs.

Clonal BCR Avidities in Late GCs Vary Substantially

To investigate the range of BCR fitness tolerated within GCs, we identified several (n = 24) B cell clones from day 16 GCs defined by shared H- and LCDR3 sequences (Figure 5). Clone 1 comprised seven descendants from a computationally inferred unmutated ancestor (UA) and putative intermediates (I1, I2) (Figures 5A and S5); intraclonal AvIn values in clone 1 clustered

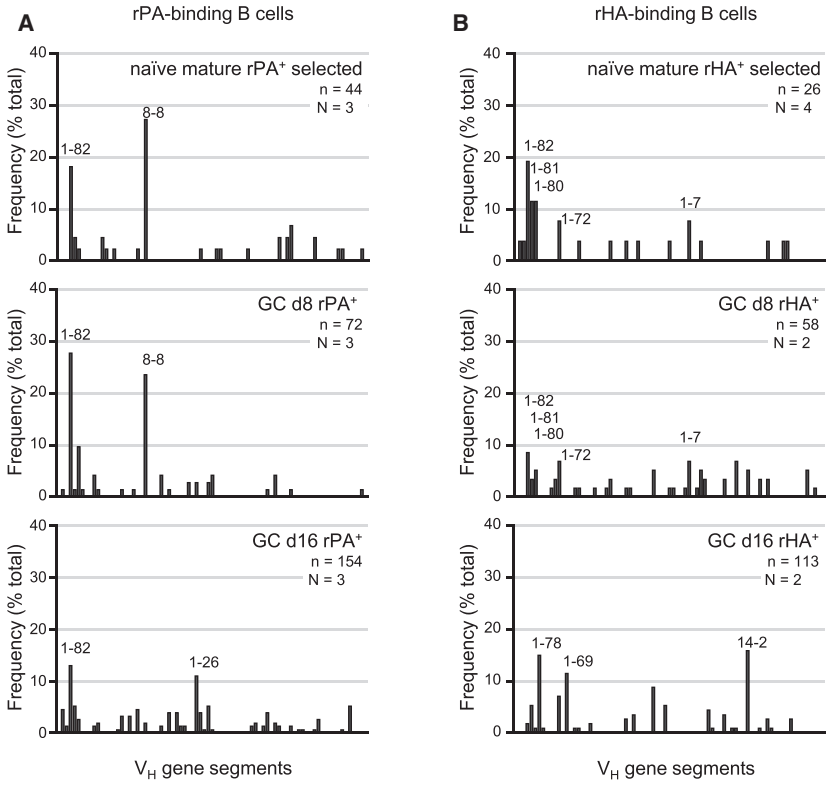

Figure 4. Clonal Diversity of Antigen-Binding B Cells Increases in Late GCs

Distributions of V_H gene segment usage among rPA-binding (A) or rHA-binding (B), naive, mature B, and GC B cells. The percentage of V_H gene segments among all V_H gene segments within each B cell subset is shown. Abbreviations are as follows: N, number of mice; n, number of sequences. The V_H gene segments frequently recovered from selected, naive, mature B cells, and day 8 and day 16 GC B cells are indicated. Combined data from two or more independent experiments are shown. See also Table S1.

about the peak of the rPA BCR avidities (0.089 to 1.5) over a 17-fold range (Figure 5A).

In contrast, rPA BCR avidities within clone 2 (six members) from the same LN fell into the lower tail of the rPA AvIn distribution (Figure 5B). AvIn in clone 2 ranged from 0.001 to 0.042, a 40-fold range, and three members showed no measurable rPA binding (Figure 5B). All members of clone 2 shared five nucleotide substitutions in their common V_H and V_L rearrangements and it was likely that these B cells represented a single GC. AvIn values between clone 1 and clone 2 differed by >100-fold, demonstrating that GC responses in a single LN were remarkably tolerant for wide ranges of BCR affinity.

We also identified several rHA specific clones from day 16 GCs (n = 19); clone 3 had 27 members with intraclonal AvIn values ranging from 0.034 to 1.5 (44-fold) (Figures 5C and S5). The two major branches from I1 held clonal members with distinct avidity distributions. Although AvIn differed only by 4-fold among the descendants of I3, AvIn values differed ~38-fold within the lineage descended from I2 (Figure 5C). Clone 4, from the same LN, comprised 13 members with AvIn some 100-fold lower than those of clone 3 (Figure S5). We conclude that interclonal selection within GCs from single LNs commonly results in BCR avidities that vary 100-fold whereas distributions of intraclonal BCR avidities might differ by only 10-fold (4- to 40-fold).

GC B Cell Responses to Unidentified Antigen also Elicit SHM and Clonal Expansion

Despite 3-log enrichment for antigen-binding B cells in GCs (Table 1), a large number of clonal IgGs derived from single GC B cells did not bind detectably to native immunogen (Table 1). This non-binding population was a majority in day 8 GC populations (70%–75%) and decreased to half (45%–50%) by day 16. The substantial frequencies of "unspecific" B cells from GCs were derived from robust and characteristic GC responses (Figure S2) and were not associated with lower IgG secretion in vitro (IgG concentrations: 0.8–2.6 μg/mL for antigen-binding GC B cells versus 0.9–2.7 μg/mL for non-binding cells).

GC B cells that did not bind immunogen accumulated V(D)J mutations at similar rates and with similar R:S ratios as did antigen-binding GC B cells (Figures 3 and 6). They formed clonal lineages indistinguishably from antigen-binding cells (Figure S6), indicating that some unknown antigen(s) or non-native form of immunogen (dark antigen) drove this substantial GC B cell population.

To determine whether non-binding IgGs might be specific for native epitopes that were lost when rPA or rHA was adsorbed to ELISA plates, we tested a subset (n = 1,116) of antigen-binding and non-binding IgGs from early and late GC B cells for their binding to soluble, native protein in a reverse ELISA (Table S4). By this method, we observed a small increase (≤7%) of antigen-binding clonal IgGs (Table S4), but the remaining GC BCR specificities could not be determined.

Injection of alum adjuvant alone did not elicit GC-like responses (Figure S7), and V_H rearrangements recovered from "unspecific" B cells in early GCs were as biased—but for different gene segments—as were those from antigen-binding GC B cells (compare Figures 4 and 6). V_H bias among "unspecific" GC B cells differed after immunization with rPA or rHA (Figure 6), suggesting that these GC B cells were not responding to common autoantigens released as a consequence of immunization or the GC response. Because it remained possible that immunization with rPA or rHA elicited distinct sets of autoreactive B cells (Sabouri et al., 2014), we assayed a subset (n = 421) of unspecific clonal IgGs from day 8 and day 16 GC B cells for reactivity to 11 autoantigens commonly associated with autoimmune diseases. Of the tested IgGs, only two (2/421; 0.5%) bound at significant levels to an autoantigen (RNP; Figures 6E and 6F). This component of the GC response might arise in response to altered forms of the rHA or rPA immunogens, generated by the

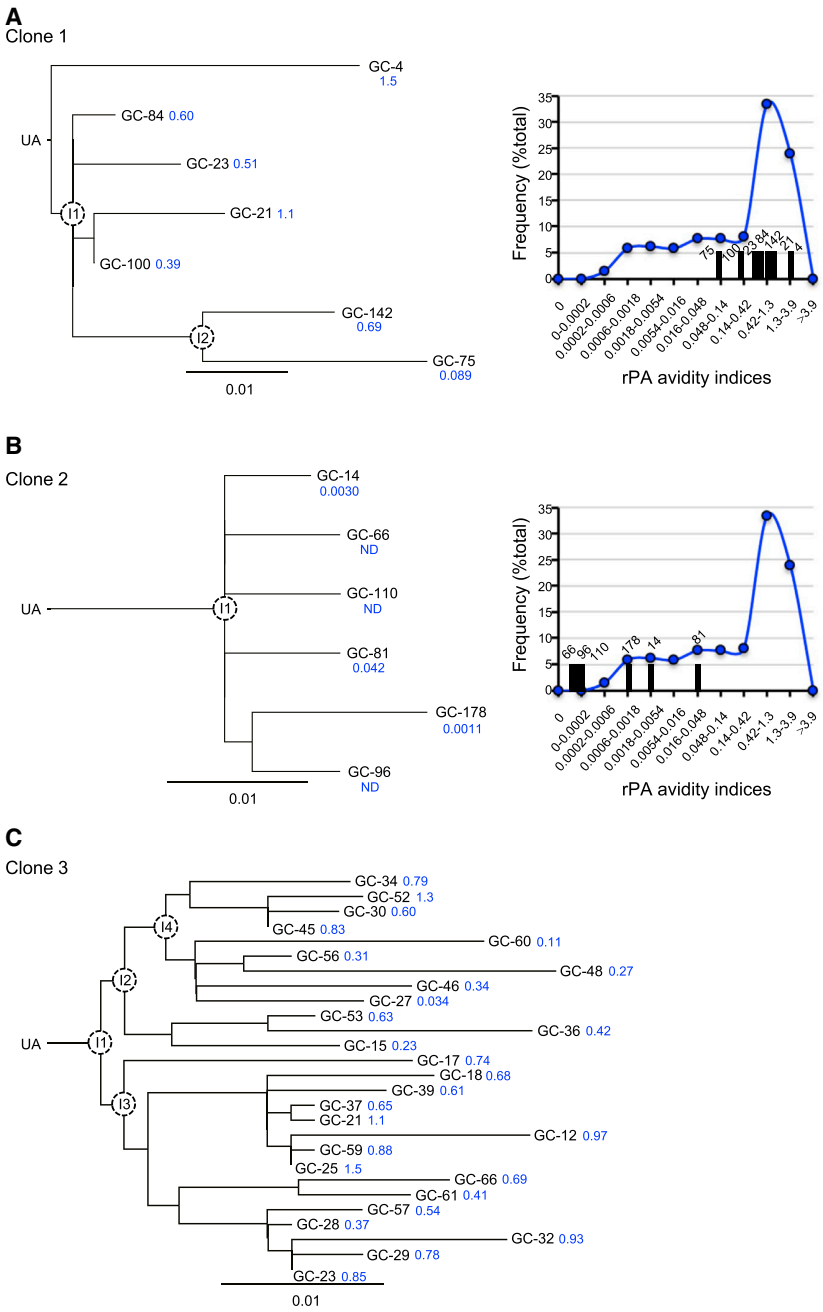

Figure 5. Clonal Genealogy by V(D)J Sequences of GC B Cells Reveals a Wide Range of Clonal BCR Avidities

These clones were identified among day 16 GC B cells (A and B, rPA GC B cells from the same LN; C, rHA GC B cells).

(A) Clone 1. Members of this clone use V_H1-50/J_H4 and Vκ1-110/Jκ2 rearrangements.

(B) Clone 2. Members of this clone use V_H1-62-2 or 1-71/D_H1-1/J_H1 and Vκ14-111/Jκ4 rearrangements. The rPA AvIn values are indicated for each member of these clones. GC-66, GC-96, and GC-110 did not show a binding to rPA at a detectable level (indicated as ND).

(C) Clone 3. Members of this clone use V_H1-26/D_H4-1/J_H3 and Vκ10-96/Jκ2 rearrangements. The unmutated ancestor was inferred via Cloanalyst (Kepler, 2013) and the maximum-likelihood phylogenetic tree was inferred via DNAML (Felsenstein, 2005). The rHA AvIn values are indicated for each member of this clone. Analyses represent two or more independent experiments. See also Figure S5.

immunization procedure or by physiological processes in vivo, as discussed more completely below.

DISCUSSION

Models of clonal selection and affinity maturation in the GCs largely depend on B cell responses in which genetically restricted B cells compete for a single epitope (Dal Porto et al., 2002; Jacob et al., 1993; Schwickert et al., 2011; Shih et al., 2002). Humoral responses to vaccines or infections instead represent clonally diverse B cells responding to polyepitopic antigens and both intraclonal competition for single epitopes and interclonal competition for distinct epitopes. To understand B cell selection to vaccines and infections, we must study B cell responses to complex antigens. We characterized the population dynamics of humoral responses to rPA and rHA and determined, at the level of individual B cells, the BCR specificity and avidity for thousands of cells from the pre-immune MF and GC B cell compartments.

Nojima cultures efficiently support the proliferation, plasmacytic differentiation, and IgG secretion of MF B cells. The cloning efficiency (~80% for mature B cells) and unbiased recovery of V_H and V_κ gene segments from unselected MF B cells indicate that these cultures provide representative samples of BCR diversity. In addition, the cultures also support IgG secretion from descendants of single GC B cells, albeit with lower cloning efficiency (~35%) and lower secreted IgG concentrations. The lower cloning efficiency for mouse GC B cells is consistent with the fragility of GC B cells in vitro (MacLennan, 1994). Nonetheless, Nojima cultures of single GC B cells from BALB/c mice immunized with NP-Ova captured all the hallmarks of the NP^a response, demonstrating that cultured B cells accurately sample the BCR repertoires of both resting and antigen-activated B cells regardless of specificity or avidity.

By determining AvIn for individual B cells, we could follow antigen-driven selection in B cell populations from their initial recruitment through the GC reaction. AvIn values for clonal IgG in culture supernatants were highly correlated ($R^2 \geq 0.88$; $p \leq 0.017$) with K_D values determined for homologous rIgG. After immunization, median AvIn values increased significantly (average,

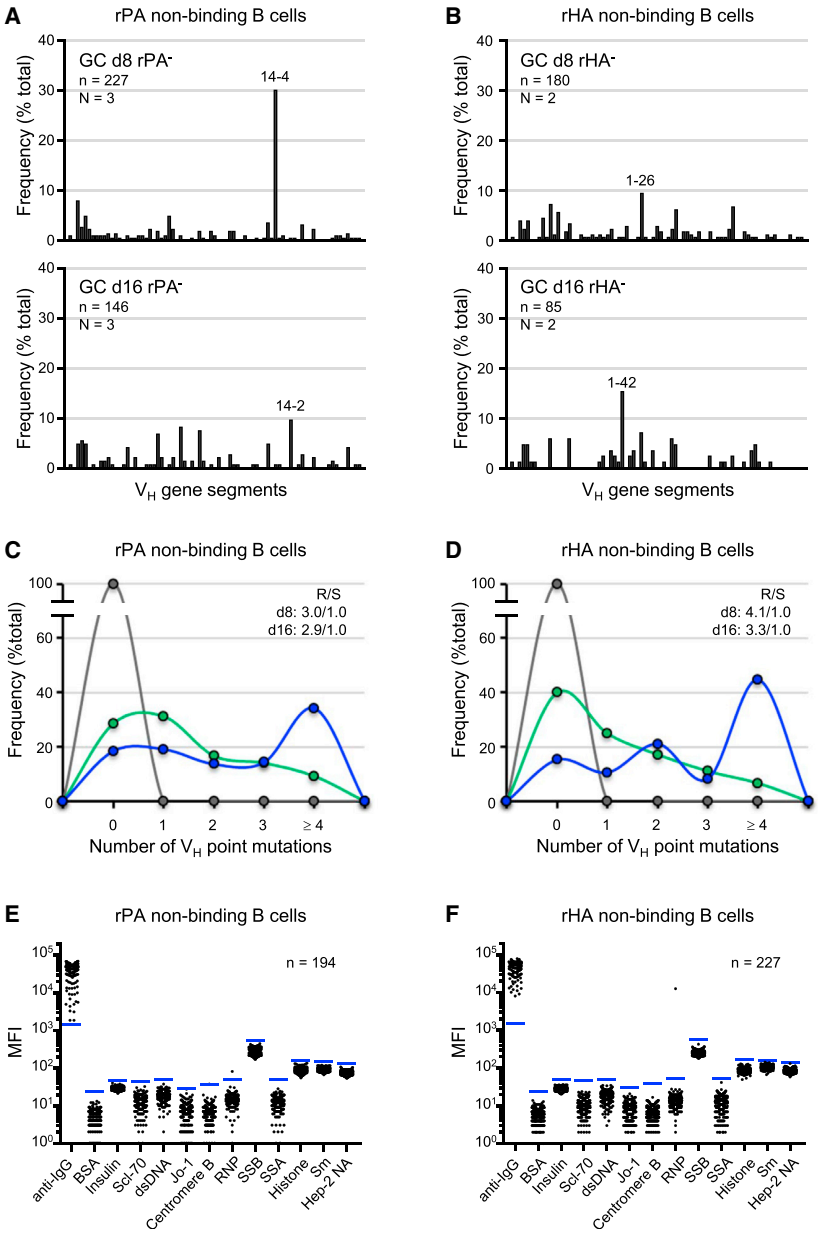

Figure 6. Unspecific GC B Cells Exhibit V_H Selection and SHM

Distributions of the V_H gene segments and V_H mutations of "unspecific" GC B cells are shown.

(A and B) The percentage of V_H gene segments among all V_H gene segments within each B cell subset is shown. Abbreviations are as follows: N, number of mice; n, number of sequences. The V_H gene segments frequently recovered from day 8 and day 16 GC B cells are indicated. See also Table S1.

(C and D) Distributions of the number of V_H point mutations in unselected MF B cells (gray; appear in both C and D; n = 125), and day 8 (green; n = 227 and 180 for rPA and rHA GCs, respectively) and day 16 (blue; n = 146 and 85) GC B cells are shown. Abbreviation is as follows: R/S, ratio of replacement/silent mutations.

(E and F) Self-reactivity of clonal Abs from unspecific GC B cell cultures (n = 194 and 227) were tested in Luminex multiplex assay. Each dot represents an individual test for each antigen. Bars in blue indicate the threshold mean fluorescence intensities (MFIs) for each antigen (average + 6 SD of B cell negative, mock-treated samples). Combined data from two or more individual mice are shown. See also Figures S6 and S7.

100-fold) during the transition from antigen-binding, MF B cells through the early GC B cell stage and into late GC B cells. The changing distributions of BCR avidities over the 16-day course of the response charted affinity maturation to rPA and rHA. For example, the near-Gaussian distribution of AvIn for rPA-binding, pre-immune B cells was consistent with the random generation of rPA BCRs; increasing BCR avidities in early GC B cells probably represented avidity-dependent selection for GC entry (Schwickert et al., 2011) and/or interclonal competition in early GC driven by germline BCR affinities (Jacob et al., 1993). The bimodal distribution of BCR AvIn among early rPA GC B cells and the broader AvIn distributions in early rHA GC B cells demonstrated recruitment of GC B cells over a broad range of BCR affinities and, probably, diverse epitopic specificities. The subsequent resolution of these broad distributions into a single high-avidity peak obviously represented affinity-dependent clonal selection, but the substantial, low-avidity "tails" of AvIn distributions in late GCs indicated that despite rapid and effective affinity maturation, GC responses were permissive for the continued survival of B cells with lower-affinity BCR. The fate of these less-fit GC B cells, especially if they enter memory compartments, will be important to determine.

The persistence of low-affinity B cells in GCs could be estimated by the fraction of the AvIn distribution not included in the high-avidity peak; conservatively, this accounted for 27% of the B cell population in GCs driven by rPA and 22% for GC B cells elicited by rHA. Although these BCR avidity distributions were complex, a simple selection model accurately described them. In brief, we propose that GC B cell proliferation is certain when BCR affinity exceeds some threshold, but lower BCR affinities induce proliferation, albeit less efficiently and in proportion to antigen-binding strength. We note that BCR avidity might be a proxy for other interactions, e.g., productive encounters with Tfh cells; our wish is to emphasize that intense GC selection is compatible with plausible biological processes that enable lower-affinity B cells to participate late into humoral responses.

AvIn varied more than two orders of magnitude among rPA and rHA GC clones. The wide range of interclonal avidity distributions suggested that any Ab feedback to establish a "systemic selection threshold" (Zhang et al., 2013) among GCs was weak

or absent during primary humoral responses, because inter- and intraclonal AvIn could vary 100-fold and 40-fold, respectively. We offer two explanations for the surprising breadth in GC BCR avidities. If GC B cells compete only locally, within the same GC (Jacob and Kelsoe, 1992), local B cell competition, as determined by intraclonal avidities, is permissive for BCR affinities that differ by one log, but not two. On the other hand, our study might overestimate intraclonal avidity differences if independent GCs arise from clonally related B cells (Jacob and Kelsoe, 1992). For example, clonal lineages with branches joined by an early intermediate that differs little from the UA could represent the progeny of a single, antigen-activated UA that seeded independent GCs. If BCR avidities varied less within branches than between branches, the actual distribution of BCR affinities tolerated within single GCs is probably below our estimate of 40-fold. We note, however, that this approach does not significantly reduce intraclonal BCR avidity differences in the GC B cell lineages presented. Even if clone 3 represented two independent GCs founded by I2 and I3, the progeny of I3 still exhibited AvIn values that differed 38-fold. Analysis of B cells from single GCs will allow us to distinguish these alternatives.

Despite the enrichment of antigen-binding B cells and substantial affinity maturation in GCs, IgG Abs from ~70% of day 8 GC cells and ~50% of day 16 GC cells did not show measurable binding to the native immunogen. What are these "unspecific" GC B cells? We can exclude B cell activation by alum alone, because the induction of GCs required rPA or rHA and because distinct patterns of V_H bias were present in the specific and "unspecific" GC B cell populations elicited by rPA or rHA; unspecific GC B cells were somehow selected by an antigen-specific process. Although each antigen, by virtue of distinct patterns of cross-reactivity, might select for different sets of autoreactive B cells (Sabouri et al., 2014), we did not detect self-reactivity among the unspecific GC B cells.

The most likely explanation for frequent unspecific GC B cells is responses to non-native conformations of the immunogen. These could be produced by dissociation or unfolding by adjuvant or by in vivo processes, e.g., cryptic epitopes exposed by degradation, neoepitopes by complement fixation, etc. Haptens, the epitopes followed in most studies of GC reactions, resist such modifications, masking the large numbers of unspecific but affinity-matured B cells we observed. That most of the unspecific GC B cells were clonally unrelated to antigen-binding GC B cells was consistent with this interpretation. Both antigen-binding and non-binding GC B cells acquired selected V_H mutations at comparable rates, indicating that both populations were similarly active in the GC reaction and excluding the possibility that non-binding GC B cells were naive immigrants. Some "unspecific" GC B cells could be very-low-affinity B cells (Dal Porto et al., 1998; Di Niro et al., 2015), but genetic evidence for strong BCR selection among these cells made this explanation unlikely. We assessed the possibility of denaturation by adjuvant by adjuvanting rPA with Alhydrogel, a pre-formed alum preparation that does not require pH-driven co-precipitation of antigen. Despite the milder conditions, 50% of day 16 GC B cells elicited by rPA absorbed in Alhydrogel failed to bind rPA (not shown).

We do not know the molecular characteristics of the antigen displayed within GCs, especially during infection. Most virus-specific Abs bind virus fragments or degraded viral proteins (Hangartner et al., 2006). Similar processes might drive the massive but "promiscuous" humoral responses to *S. enterica* infections (Di Niro et al., 2015). We showed here that significant GC responses to non-native antigen forms did not require the inflammatory and apoptotic milieu of infection. Although we think the possibility remote, we cannot rule out that the true frequency of unspecific GC B cells differs from our estimates. Nonetheless, these cells certainly were present and viable, expressed a canonical GC phenotype, and showed evidence of V(D)J mutation and selection. Whether they are less frequent or more, the identification of GC B cells that undergo somatic evolution without measurable affinity for native immunogen has important implications for understanding the population dynamics of GCs and leads to a wide range of future experiments in both basic and translational immunology.

In conclusion, we demonstrated that it was possible to detail humoral responses to complex protein antigens at the level of individual cells. Our methods provide a cost-effective and accessible alternative to cloning and re-expressing paired H- and L-chain rearrangements from isolated, single B cells (Wardemann et al., 2003; Wrammert et al., 2008). Immunization with rPA or rHA selectively recruited antigen-binding, mature B cells into GCs and there drove SHM and affinity maturation. In addition, our experiments revealed aspects of the GC reaction that could not be gleaned from responses to haptens. GC reactions against complex protein antigens became increasingly diverse as B cell clones that were rare initially became frequent in late GCs. This increasing clonal diversity is consistent with other analyses of clonal diversity after immunization with influenza (Kavaler et al., 1991) and cannot represent the immigration of naive B cells, because the V(D)J rearrangements of these latecomers were substantially mutated. If these later, highly fit populations represented GC B cell immigration from distal sites, this migration would homogenize affinity-driven selection across GCs, rather than diversify it. Moreover, intraclonal BCR avidities in single lymph nodes might vary 40-fold and interclonal avidities, even on day 16 of primary responses, might differ by several orders of magnitude. This latter observation is at odds with any global mechanism for affinity selection.

A potential benefit of increased clonal diversity in late GCs might be the generation of an expanded BCR repertoire that responds rapidly not only to homologous challenges but also to structurally related antigens. For example, this mechanism could provide accelerated humoral responses to infection by a hetrosubtypic influenza virus (Baumgarth, 2013). This broadening of the BCR repertoire would be aided by the surprising prevalence of widely different BCR avidities among GC B cells. The apparent selective advantage of such features of the GC reaction suggests that there are mechanisms to be discovered that reinforce and preserve them.

EXPERIMENTAL PROCEDURES

Mice and Immunizations

Female C57BL/6 and BALB/c mice were obtained from the Jackson Laboratory and maintained under specific-pathogen-free conditions at the Duke University Animal Care Facility. 8- to 12-week-old mice were used in this study. C57BL/6 mice were immunized with alum alone or with 20 μg of rPA (BEI Resources) or rHA (see below) precipitated in alum via footpad. BALB/c mice were immunized with 10 μg of NP-Ova precipitated in alum via base-of-tail.

In some experiments, we mixed rPA with Alhydrogel and immunized mice with Alhydrogel and rPA mixtures to characterize GC responses (Cain et al., 2013). All experiments involving animals were approved by the Duke University Institutional Animal Care and Use Committee.

Expression and Purification of HA

The rHA for the full-length, soluble ectodomains of H1 A/Solomon Islands/03/2006 was cloned, expressed, and purified as previously described (Schmidt et al., 2015; Whittle et al., 2011). Detailed methods are described in Supplemental Experimental Procedures.

Flow Cytometry

GC B cells (GL-7$^+$B220^{hi}Fas$^+$IgD$^-$ or GL-7$^+$B220^{hi}CD38^{lo}IgD$^-$CD93$^-$CD138$^-$), control mature B cells (GL-7$^-$B220^{hi}CD38^{hi}IgD$^+$CD93$^-$CD138$^-$), and unselected MF B cells (B220^{hi}CD93$^-$IgM^{int}IgD^{hi}CD21^{int}CD23^{hi}) were identified as described (Kuraoka et al., 2011). GC B cells and control mature B cells were sorted from popliteal LNs of immunized or naive mice. Unselected MF B cells and antigen-binding, mature B cells (rPA$^+$ or rHA$^+$, B220^{hi}CD93$^-$CD38^{hi}IgL$^+$) were sorted from spleens of naive mice. Fluorochrome or biotin-tagged rPA or rHA was prepared with labeling kit (Dojindo or Pierce). The rHA tetramer was a gift of M.A. Moody (Duke University). Labeled cells were analyzed/sorted in a FACS Canto (BD Bioscience) or a FACS Vantage with DIVA option (BD Bioscience). Flow cytometric data were analyzed with FlowJo software (Treestar). Doublets were excluded by FSC-A/FSC-H gating strategy. Cells that take up propidium iodide were excluded from our analyses.

NB-21 Feeder Generation

NB-21 feeder cells were established by introducing mouse IL-21 (mIL-21) cDNA into 40LB cells (Nojima et al., 2011) by retroviral transduction. After transduction, 40LB cells that expressed human CD8α, a marker for transduction, were sorted by a FACS Vantage. We subcloned sorted feeder cells (NB-21) by limiting dilution and established a clone, which efficiently supported proliferation and differentiation of single MF B cells to secrete large amounts of IgG. We named this clone as NB-21.2D9 and used it for all experiments. Detailed methods are described in Supplemental Experimantal Procedures.

Single B Cell Culture

Single B cells were cultured in the presence of NB-21.2D9 feeder cells. NB-21.2D9 cells were seeded into 96-well plates at 1,000 cells/well in B cell media (BCM); RPMI-1640 (Invitrogen) supplemented with 10% HyClone FBS (Thermo Scientific), 5.5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, and MEM nonessential amino acid (all Invitrogen). Next day (day 0), recombinant mouse IL-4 (Peprotech; 2 ng/mL) was added to the cultures, and then single B cells were directly sorted into each well by a FACS Vantage. On day 2, 50% (vol.) of culture media were removed from cultures and 100% (vol.) of fresh BCM were added to the cultures. On days 3 to 8, two-thirds of the culture media were replaced with fresh BCM every day. On day 9 or 10, culture supernatants were harvested for ELISA determinations and culture plates were stored at −80°C for V(D)J amplifications.

Determinations of Total and Antigen-Specific IgG and BCR AvIn

Presence of both total and antigen-specific IgG in culture supernatants were determined by ELISA or Luminex multiplex assay. BCR AvIn can be considered a measure of specific activity determined by antigen-binding capacity/IgG concentration in reference to monoclonal standards: BAP0105 for rPA (70×10^{-9} M; Abcam) and "musinized" CH67 for rHA (2.4×10^{-9} M). The specific activity for each standard was determined as a binding ratio, bound IgG_{Ag}/bound IgG_{total}, and defined as equal to 1.0. For each culture supernatant sample, concentrations of total IgG and antigen-binding IgG were determined in reference to monoclonal standards. AvIn, the ratio of bound IgG_{Ag}/bound IgG_{total} of each sample, therefore represent the antigen-binding capacity of each clonal IgG relative to the reference standard at the same concentrations. For example, AvIn of 1.0 and 0.3 represent clonal IgGs with antigen-binding activity (our estimate of avidity) equal to 100% and 30% of the standard, respectively. These procedures are detailed in Supplemental Experimental Procedures.

BCR Repertoire Analysis and *Ig* SHM

V(D)J rearrangements of cultured B cells were amplified by a nested PCR. In brief, total RNA was extracted from selected samples using TRIzol or TRIzol LS reagents (Invitrogen). cDNA was synthesized from DNase I-treated RNA using Superscript III with oligo $(dT)_{20}$ primers. One twentieth (volume) of the cDNA was then subjected to two rounds of PCR using Herculase II fusion DNA polymerase (Agilent Technologies) with established primers (Rohatgi et al., 2008; Tiller et al., 2009). Primary PCR: 95°C for 4 min, followed by 2 cycles of 95°C for 30 s, 64°C for 20 s, and 72°C for 45 s; 3 cycles of 95°C for 30 s, 62°C for 20 s, and 72°C for 45 s; and 25 cycles of 95°C for 30 s, 60°C (for IgH) or 52°C (for Igκ) for 20 s, and 72°C for 45 s. Secondary PCR: 95°C for 4 min, followed by 40 cycles of 95°C for 30 s, 60°C (for IgH) or 45°C (for Igκ) for 20 s, and 72°C for 45 s. V(D)J amplicands were gel purified, ligated into vectors, and transformed into bacteria (McWilliams et al., 2013). DNA sequences were obtained at Duke DNA sequencing facility. The rearranged V, D, and J gene segments were first identified using IMGT/V-QUEST (http://www.imgt.org/), and then numbers and kinds of point mutations were determined.

Statistics

Statistical significance ($p < 0.05$) was determined by two-tailed Student's t test and Mann-Whitney's *U* test.

ACCESSION NUMBERS

V(D)J sequence data of unselected MF, rPA-, or rHA-binding MF, day 8 and day 16 GC B cells are available at GenBank (www.ncbi.nlm.nih.gov/GenBank), accession numbers KU256230–KU257459.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.02.010.

AUTHOR CONTRIBUTIONS

M.K. and G.K. designed research. M.K., A.G.S., T.N., F.F., and A.W. performed research. D.K. provided reagents. T.B.K. developed mathematical models. M.K., A.G.S., T.N., F.F., A.W., S.C.H., T.B.K., and G.K. analyzed data. M.K., S.C.H., T.B.K., and G.K. wrote the paper. G.K. directed the study.

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