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Naïve B cells with high-avidity germline-encoded antigen receptors produce persistent IgM+ and transient IgG+ memory B cells

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SUMMARY

Although immune memory often lasts for life, this is not the case for certain vaccines in some individuals. We sought a mechanism for this phenomenon by studying B cell responses to phycoerythrin (PE). PE immunization of mouse strains with *Igh*^{b} immunoglobulin (Ig) variable heavy chain (V_H) genes elicited affinity-matured switched Ig memory B cells that declined with time, while the comparable population from an *Igh*^{a} strain was numerically stable. *Igh*^{b} strains had larger numbers of PE-specific naïve B cells, generated smaller germinal center responses, and larger numbers of IgM memory cells than the *Igh*^{a} strain. The properties of PE-specific B cells in *Igh*^{b} mice correlated with usage of a single V_H that afforded high-affinity PE binding in its germ-line form. These results suggest that some individuals may be genetically predisposed to generate non-canonical memory B cell responses to certain antigens because of avid antigen binding via germ-line encoded V_H elements.

eTOC

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AUTHOR CONTRIBUTIONS

K. A. P. designed the study, did experiments, analyzed data, and wrote the manuscript, R. W. M. designed the study, did experiments, and analyzed data, A. S. P. did experiments, P. J. G. designed the study and provided discussion, M. K. J. designed the study, analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS

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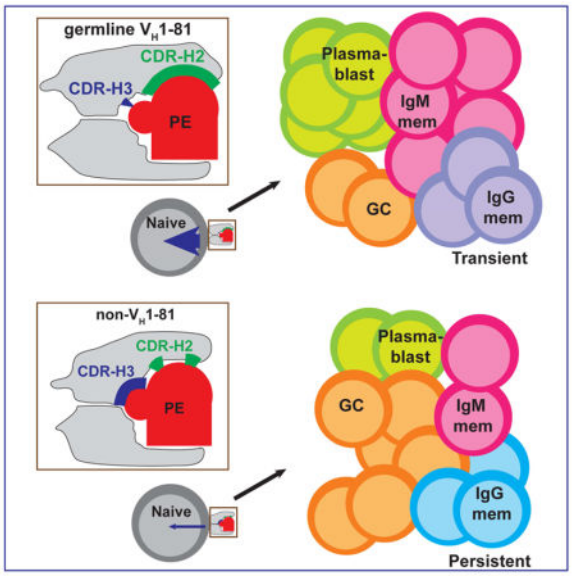
Immunity induced by certain vaccines declines over time. By studying B cell responses to phycoerythrin, *Pape et al.* find that memory B cells can be short-lived when generated from precursors that experience unusually strong early signals through their un-mutated antigen receptors.



Keywords

V_H; memory B cell; naïve B cell; repertoire

INTRODUCTION

Antigen-specific immune memory results from the activation of naïve B cells. Usually, a naïve B cell recognizes an epitope on an antigen using six complementarity determining regions (CDR) of its immunoglobulin (Ig) heavy (H) and light (L) chain B cell receptor (BCR). Although germ-line encoded CDR1 and CDR2 contribute, CDR3s composed of the joints between variable (V), diversity, and joining segments of the IgH and IgL chains are generally the most important determinants of antigen binding (Xu and Davis, 2000). Antigen binding and signals from helper T cells cause rare naïve B cells to proliferate and differentiate into short-lived antibody-secreting plasmablasts or germinal center cells, some of which switch their IgM constant region to IgG and acquire somatic mutations in the V region (McHeyzer-Williams and McHeyzer-Williams, 2005; Tangye and Tarlinton, 2009). Cells that acquire mutations that improve antigen binding gain a survival advantage by outcompeting other B cells for T cell help and emerge from the germinal center reaction as plasma cells or memory cells (Victora and Nussenzweig, 2012) capable of generating rapid secondary responses (Dogan et al., 2009; McHeyzer-Williams et al., 2015; Pape et al., 2011; Yoshida et al., 2010).

Memory B cells are generally very long-lived. For example, murine memory B cells specific for nitrophenyl or hen egg lysozyme show no reduction in number over a lifetime (Jones et

al., 2015; Weisel et al., 2016) and human memory B cells specific for the smallpox vaccine are numerically stable for 50 years (Crotty et al., 2003). This rule, however, does not apply to all immune responses. Human B cells specific for a conserved epitope on the influenza hemagglutinin stem region declined dramatically over a 70-week period after booster vaccination (Wheatley et al., 2015), while non-stem-specific memory B cells were stable. Memory cells specific for sheep red blood cells (Dogan et al., 2009) or malaria merozoite surface protein 1(Krishnamurty et al., 2016) slowly decline in mice after immunization. Similarly, C57BL/6 (B6) mice immunized with the algal protein phycoerythrin (PE) produce unstable swIg memory cells that decline with a half-life of about 80 days (Pape et al., 2011). Gitlin et al. (Gitlin et al., 2016) showed that the decline of PE-specific swIg memory B cells is related to the acquisition of somatic mutations that confer polyreactivity to self-antigens. B6 mice also generated an unusually large population of PE-specific IgM memory cells, which was numerically stable for the life the mouse (Pape et al., 2011).

These results suggest that certain features of memory B cells depend on the inducing antigen. Here, however, we show that the nature of the B cell repertoire, not an inherent property of the antigen itself, can influence memory B cell formation. We confirmed that although B6 or C.B-17 mice, which share a set of *Igvh* genes, generated large numbers of PE-specific IgM memory cells and unstable swIg memory B cells, BALB/c mice, which have a different set (Collins et al., 2015; Herzenberg and Herzenberg, 1974) formed many fewer IgM memory cells and had stable swIg memory B cells. The memory B cell phenotypes observed in B6 and C.B-17 mice correlated with usage of BCRs containing a single V_H segment that afforded germline-encoded high affinity for PE. These results suggest that B cells that bind antigen avidly with germline-encoded elements of the BCR undergo a different memory B cell program than B cells that bind in the conventional CDR3-dependent manner.

RESULTS

B6 mice have more PE-specific B cells than BALB/c mice

We investigated whether the tendency of B6 mice to produce IgM memory cells and unstable swIg memory B cells (Gitlin et al., 2016; Pape et al., 2011) was shared by BALB/c and C.B-17 mice. B6 and BALB/c mice have different sets of *Ighv* genes defined as the Igh^b haplotype for B6 and Igh^a for BALB/c mice (Collins et al., 2015; Herzenberg and Herzenberg, 1974). C.B-17 (C.BKa-Igh^b/IcrCrl) mice have the Igh^b set of *Ighv* genes like B6 mice but the other genes of BALB/c mice including *H2*, *Igl,* and *Igk* (Bosma and Bosma, 1974).

We first assessed the pre-immune repertoires of PE-specific B cells in these strains using an antigen-based cell enrichment method (Pape et al., 2011; Schittek and Rajewsky, 1990; Taylor et al., 2012). Spleen and lymph node cells from each mouse were mixed with PE and another antigen allophycocyanin (APC), and then magnetic beads coated with anti-PE and APC antibodies. The cell suspensions were passed over magnetized columns to enrich the PE- and APC-bound cells. The cells in the pre- and post-enriched samples were fixed, permeabilized, and stained with lineage-defining antibodies. B cells were identified as cells lacking non-B cell lineage markers and expressing Ig and B220 (Figure 1A). Although PE-

and APC-specific B cells could be detected in the total B cell population in unenriched spleen and lymph node samples from BALB/c, B6 and C.B-17 mice, the fraction of these cells increased by about 100-fold in the enriched samples (Figure 1B). PE- and APC-specific B cells were not detected in MD4 transgenic mice (Goodnow et al., 1988) that only contained monoclonal B cells specific for hen egg lysozyme (Figure 1B). These results showed that antigen-based B cell enrichment is a sensitive and specific method for detection of the antigen-specific B cells from polyclonal repertoires.

Use of this method revealed differences between the PE-specific pre-immune B cell repertoires. As previously described (Pape et al., 2011), B6 mice that were never exposed to PE or APC contained about 20,000 PE-specific and 4,000 APC-specific naïve B cells (Figure 1C). In contrast, BALB/c mice had only about 1,400 PE-specific naïve B cells but contained a similar number of APC-specific naïve B cells as B6 mice (Figure 1C). C.B-17 mice contained about 20,000 PE-specific and 4,000 APC-specific naïve B cells like B6 mice (Figure 1C), suggesting that V_H segments encoded in the *Igh*^{b} locus accounted for the large number of PE-specific naïve B cells.

Analysis of the relative amount of antigen bound per PE-specific B cell revealed another difference between BCRs with *Igh*^{a}– or *Igh*^{b}–encoded IgH chains. PE-specific naïve B cells from B6 or C.B-17 mice with *Igh*^{b}–encoded IgH chains bound 2–3-fold more PE on average than PE-specific naïve B cells from BALB/c mice with *Igh*^{a}–encoded IgH chains (Figure 1B, 1D). This difference was not due to differences in BCR density since the antigen binding levels were normalized to the levels of immunoglobulin associated-beta (CD79b) expression. Thus, V_H segments encoded in the *Igh*^{b} locus also accounted for the capacity of naïve B cells to bind PE with higher affinity than V_H segments encoded in the *Igh*^{a} locus of BALB/c mice.

The PE-specific repertoires in *Igh*^{a} and *Igh*^{b} mice produce different primary immune responses

The different mouse strains were then immunized with PE in complete Freund's adjuvant (CFA) to assess the primary immune response. As shown in Figure 2A, immunization caused marked expansion of PE-specific B cells, which peaked on day 9 at about 10^6 cells in BALB/c, B6, and C.B-17 strains. Correction for the starting number of cells in each population revealed that PE-specific B cells paradoxically expanded better on average in BALB/c (*Igh*^{a}) (300-fold) than in B6 or C.B-17 (*Igh*^{b}) mice (50-fold). The number of PE-specific B cells then declined in all strains after the peak (Figure 2A) before flattening out at about day 100 at 10^5 cells in B6 and C.B-17 (*Igh*^{b}) mice and 10^4 in BALB/c (*Igh*^{a}). Thus, by the end of the primary response, B6 and C.B-17(*Igh*^{b}) mice generated 10-times more PE-specific B cells than BALB/c (*Igh*^{a}), which correlated with the size of the respective naïve cell populations.

Germinal center B cells and plasmablasts were then measured to assess the quality of the primary response generated in each strain. The GL-7 marker was used to identify germinal center cells and abundant intracellular Ig was used to identify plasmablasts. The frequency and total numbers of PE-specific plasmablast and germinal center cells differed between the *Igh*^{a} and *Igh*^{b} strains at the peak of the response on days 9–16. The PE-specific population in

BALB/c (*Igh*^{a}) mice consisted of about 80% germinal center cells and 5% plasmablasts, while the population in B6 and C.B17 (*Igh*^{b}) mice consisted of about 45% germinal center cells and 10% plasmablasts (Fig. 2B). BALB/c (*Igh*^{a}) mice made 30,000 PE-specific plasmablasts while B6 and C.B17 (*Igh*^{b}) mice generated 50,000 (Fig. 2C). Despite a smaller number of naïve precursors, BALB/c (*Igh*^{a}) mice generated 500,000 germinal center cells, which was more than the 250,000 produced by B6 and C.B-17 (*Igh*^{b}) mice. Thus, at the time of peak clonal expansion, the small PE-specific pre-immune repertoire in *Igh*^{a} mice generated fewer plasmablasts and more germinal center cells than the large repertoire in *Igh*^{b} mice.

It was possible that PE-specific B cells in *Igh*^{a} mice produced an increased frequency of germinal center cells because they experienced less clonal competition than more numerous PE-specific B cells in *Igh*^{b} mice. This issue was addressed by transferring CD45.1$^+$ naïve B cells from *Igh*^{a} mice into CD45.1$^-$ *Igh*^{b} mice, thereby creating a situation where PE-specific *Igh*^{a} B cells had to compete with much more numerous PE-specific *Igh*^{b} B cells. The recipient mice were immunized with PE in CFA and PE-specific B cells of donor or recipient origin were analyzed 17 days later. As shown in Fig. 2D, PE-specific *Igh*^{a} B cells produced a higher percentage of germinal center B cells than PE-specific *Igh*^{b} B cells even when competing with these B cells in the same mice. Therefore, overproduction of germinal center B cells was not due to clonal competition and more likely an intrinsic property of PE-specific *Igh*^{a} B cells.

Igh^a mice produce fewer IgM memory cells and more stable swIg memory cells than *Igh*^{b} mice

Analysis of PE-specific memory B cells revealed another difference between the strains. Memory cells were identified as GL-7$^-$ intracellular Ig^{low} cells (Figure 2B) that expressed IgM and IgD or lacked these isotypes and thus must have expressed a switched isotype (Figure 3A). BALB/c (*Igh*^{a}) mice generated about 30,000 PE-specific IgM and swIg memory cells 36 days after immunization, which fell to about 10,000 cells in each population by day 100 (Figure 3A and B). Both populations were then maintained at this number until day 400 (Figure 3A and B). As previously described (Gitlin et al., 2016; Pape et al., 2011), B6 mice (*Igh*^{b}) mice generated about 200,000 PE-specific IgM and 50,000 swIg memory cells 36 days after immunization, which fell to about 100,000 and 20,000 cells, respectively, by day 100 (Figure 3A and B). The IgM memory cells were maintained at 100,000 cells per mouse until day 400, while the swIg memory cells declined from 20,000 to 2,000 (Figure 3A and B). The numbers of PE-specific IgM and swIg memory cells in C.B-17 mice were like those in B6 mice although the analysis of C.B-17 mice was only carried out until day 200. (Figure 3B). Together the results showed that large populations of IgM memory cells and declining swIg memory cells were features of mice containing the *Igh*^{b} set of *Ighv* genes.

The *Ighv1-81* gene segment encodes most PE-specific V_H chains in *Igh*^{b} mice

We then considered the possibility that the differences in the PE-specific B cell responses in *Igh*^{a} and *Igh*^{b} mice were due to differences in BCR binding to PE. We assessed *Ighv* gene usage of PE-specific B cells in the two strains as a first step toward testing this idea. Naïve

and memory PE-specific B cells were purified from BALB/c (*Igh*^{a}), B6 (*Igh*^{b}), or C.B-17 mice (*Igh*^{b}) and *Ighv* sequences were amplified using a common variable region primer and specific constant chain reverse primers. Hundreds of unique clones were examined from multiple mice of each strain.

The analysis had to account for the fact that the sets of *Ighv* genes *in Igh*^{a} and *Igh*^{b} are different. Strains such as B6 and C.B-17 have 99 functional *Ighv* genes, which have been annotated by The International Immunogenetics Information System (IMGT). *Igh*^{a} strains such as BALB/c have 164 *Ighv* genes, only 5 of which are completely conserved in *Igh*^{b} strains. Although many of the remaining 159 *Ighv* genes in BALB/c mice have homologs in B6 mice, the BALB/c versions have some nucleotide differences from the B6 homologs and have not been annotated by IMGT.

We found that 50–90% of the naïve B cells in the PE-specific populations of unimmunized B6 and C.B-17 *Igh*^{b} mice used one *Ighv* gene, *Ighv1-81* (Figure 4A and Table S2 and S3). This *Ighv* gene is not present in *Igh*^{a} strains (Collins et al., 2015) and was used at a frequency of only 6% in the non-PE-specific B cells from naïve B6 mice (Figure 4A and Table S4). The most common *Ighv* genes present in the naïve repertoire of PE-specific B cells in BALB/c *Igh*^{a} mice were found at a frequency of only 7% (Figure 4A and Table S1). The IgM and IgG PE-specific memory B cell repertoires of B6 and C.B-17 *Igh*^{b} mice were also dominated by *Ighv1-81*, whereas individual *Ighv* genes from the BALB/c memory cells were found at frequencies less than 10% (Figure 4B and Tables S5–10). Thus, a single *Ighv* gene was used to encode the IgH chains of most of the PE-specific naïve and memory B cells in *Igh*^{b} mice, while the IgH chains of PE-specific B cells in *Igh*^{a} mice were encoded by a more evenly distributed set of *Ighv* genes.

The fact that a single V_H was used in the IgH chains of most PE-specific BCRs in *Igh*^{b} strains raised the possibility that these BCRs bound to a single epitope. We assessed this possibility by mixing an unlabeled Fab fragment from a V_H1-81-containing PE-specific monoclonal antibody (CL33) with PE prior to staining of spleen and lymph nodes cells and magnetic bead-based cell enrichment. The rationale was that CL33 would occlude a single epitope on PE and prevent any naïve B cell with a BCR specific for that epitope from binding (Wheatley et al., 2015). Addition of CL33 reduced the number of PE-binding B naïve cells in B6 mice by about 90% but had no effect on APC-binding B cells (Figure 5A and B). Only 9% of the naïve B cells that bound PE in the presence of CL33 used V_H1-81, while 24% used V_H10-1 (Figure 5C and Table S11), which was used by only 3% of PE-specific B cells in the absence of CL33 (Table S2). Addition of CL33 reduced the number of BALB/c naïve B cells that bound PE by about 75% (Figure 5B). These results suggest that almost all V_H1-81-containing PE-specific BCRs from B6 mice and most from BALB/c mice recognize a single or overlapping epitope(s).

The dominance of cells expressing V_H1-81 IgH chains (Figure 4A) made it likely that these cells accounted for the high affinity PE binding exhibited by the population of B cells from naive *Igh*^{b} mice (Figure 1D). This contention was supported by the observation that the *Igh*^{b} naïve B cells that bound PE in the presence of CL33, which were greatly enriched for non-V_H1-81-expressing cells (Figure 5C), bound less PE per cell than the unblocked V_H1-81-

dominated population (Figure 5D). On the contrary, *Igh*^{a} naïve B cells that bound PE in the presence of CL33 bound PE as well as the unblocked population (Figure 5D). These results indicate that V_H1-81-containing BCRs from *Igh*^{b} mice bound to the CL33 epitope on PE with a higher average affinity than BCRs that bound to other epitopes on the protein. Furthermore, even though many PE-specific BCRs from *Igh*^{a} mice also bound to the CL33 epitope, these BCRs had the same low affinity as BCRs specific for other epitopes (Figure 5D).

The hypothesis that V_H1-81 IgH chains accounted for the high affinity PE binding exhibited by the population from *Igh*^{b} mice (Figure 1D) was also tested using CL33 and three other PE-specific monoclonal antibodies: CL20 (V_H1-81), CL21 (V_H8-5), and CL30 (V_H1-26). The V_H1-81-containing monoclonal antibodies CL20 and CL33 bound PE with much higher affinity than the non-V_H1-81-containing ones (Fig. 5E). Although the sample size was small, these results are consistent with V_H1-81 conferring higher affinity binding to PE than other V_H segments.

V_H1-81-containing antibodies bind to PE mainly with CDR-H2

We then analyzed the amino acid sequences of V_H1-81 chains from PE-specific BCRs to search for clues about how these receptors bind PE with high affinity. We initially focused on the third heavy-chain complementarity-determining regions (CDR-H3) formed by recombination of V, D, and J segments because these elements are normally the major determinants of antigen specificity (Xu and Davis, 2000). The CDR-H3s from total non-PE binding naïve B cells from B6 mice contained a normal distribution of amino acid lengths centering on 11 amino acids (Figure 6A) with approximately equal representation of all four J_H segments (Figure 6B). The CDR-H3s from PE-binding naïve B cells from B6 or C.B-17 mice also contained a normal distribution of lengths, although centering on 12 amino acids, and were modestly enriched for the J_H2 segment (Figure 6A and B). Therefore, CDR-H3s from PE-binding V_H1-81 BCRs were not limited to a certain amino acid length or J_H segment indicating that CDR-H3 was not a major determinant of PE binding. In contrast, the CDR-H3s from PE-specific V_H10-1 BCRs were either 6 or 12-amino acids in length and preferentially used a single J_H (J_H4) (Figure 6A and B) as expected for CDR-H3-dependent antigen-binding. These results suggest that unlike conventional IgH chains such as those containing V_H10-1, which derive much of their antigen binding energy from CDR-H3, V_H1-81 chains achieve high affinity through some other part of the protein.

The lack of evidence for antigen-driven selection of the CDR-H3s in V_H1-81-containing BCRs prompted an analysis of the CDR-H1 and CDR-H2 elements. We determined whether these regions were targeted for somatic mutations as expected for domains involved in antigen binding. On the contrary, the V_H1-81 CDR-H1 and CDR-H2s expressed by PE-specific swIg memory B cells contained about the same low number of replacement mutations as the framework regions while the CDR-H1 and especially CDR-H2s of non-V_H1-81 Igs had many more mutations (Fig. 6C). These results are consistent with the germline-encoded sequences of CDR-H1 and/or CDR-H2 of V_H1-81conferring PE binding that could not be improved in the germinal center reaction.

This possibility was examined using the PE-binding monoclonal antibodies. The CL33 and CL20 antibodies bind PE with the identical affinity (Fig. 5E) and both use V_H1-81 and fortuitously the same V_κ, V_κ 10-94 (V_H1-81-containing PE-specific antibodies use a diverse set of IgL chains (Gitlin et al., 2016)). The Igκ chains differed by only one amino acid and the CDR-H1 and CDR-H2s by 3 of 16 amino acids with only one non-conservative difference (Fig. 6D). In contrast, the CDR-H3s differed by 11 of 13 amino acids. The fact that these antibodies bind PE with identical affinity and amino acid sequences except for markedly different CDR-H3s is consistent with V_H1-81 CDR-H1 and/or CDR-H2 being more important for PE binding than CDR-H3.

Recombinant antibodies and variants containing swapped domains were then generated in 293T cells to assess the contributions of the individual CDRs to PE-binding. CL33 was used as a representative V_H1-81-containing antibody and CL30 as a non-V_H1-81 (V_H1-26)-containing antibody. These antibodies also contain different Vκ chains (Vκ 10–94 for CL33 and Vκ8–27 for CL30), and may bind their epitopes in different ways. Nonetheless, replacement of the CL33 CDR-H3 with that of CL30, which differed at 10 of the 13 amino acid positions, had almost no effect on PE-binding, while replacement with that of an ovalbumin-specific IgH, which differed at 12 of the 13 amino acid positions reduced PE-binding but did not destroy it. Thus, CL33 was remarkably tolerant to changes in its CDR-H3. In contrast, CL30 was intolerant to changes in its CDR-H3 as substitution of the CL33 CDR-H3 abolished CL30 binding to PE. PE-binding by CL30 and CL33 was abolished by replacement of their CDR-H2s with those of the other antibody. These results suggested that the V_H1-81-containing CL33 antibody bound to PE with CDR-H2 and to lesser extent, CDR-H3, while a more conventional antibody, CL30, depended on all three elements.

DISCUSSION

We found that the population of naïve polyclonal PE-specific B cells in *Igh*^{b} mice is dominated by cells expressing a single V_H, V_H1-81. Multiple lines of evidence indicate that this V_H segment confers high affinity binding to PE that is more dependent on the germline version of its CDR-H2 than conventional BCRs that depend mainly on CDR-H3 and somatic mutations. V_H1-81 CDR-H3s expressed by clonally expanded swIg memory B cells lacked a common length or J_H segment suggesting that elements other than CDR-H3 were critical for PE binding. Furthermore, the V_H1-81 CDR-H2s expressed by PE-specific swIg memory B cells contained an atypically low number of replacement mutations indicating that the germline-encoded sequence could not be improved through germinal center selection. Finally, domain-swapping experiments showed that V_H1-81-containing antibodies derive much of their binding energy from CDR-H2 with less contribution from CDR-H3, whereas conventional Igs likely derive much their binding energy from CDR-H2 and CDR-H3.

Antigen binding by a germline-encoded element likely explains the high frequency of V_H1-81-containing PE-specific naïve B cells in *Igh*^{b} mice because the probability of expressing a single V_H segment is much higher than a specific CDR-H3 generated by VDJ recombination. However, the 15,000 V_H1-81-containing PE-specific naïve B cells accounted for only a small fraction of the approximately 5 million total V_H1-81-containing B cell

population in *Igh*^{b} mice. This discrepancy is likely explained by the fact that only certain IgL chains are conducive to PE binding when paired with V_H1-81.

Is likely that CDR-H2-focused high affinity binding to PE determined the intrinsic tendency of V_H1-81-expressing naïve B cells to produce more IgM memory B cells, more plasmablasts, and fewer germinal center cells than B cells expressing other V_H segments. Strong BCR signaling has been shown to direct naïve B cells away from the germinal center B cell fate to become IgM memory cells via the germinal center-independent pathway (Taylor et al., 2012) or plasmablasts (Benson et al., 2007; Phan et al., 2006; Smith et al., 1997). It is also possible that production of unstable swIg memory B cells is an inevitable consequence of high affinity antigen binding by a germline-encoded BCR element. Nussenzweig and colleagues showed that the decline of PE-specific swIg memory B cells in B6 mice depends on somatic mutation and is associated with BCR mutations that confer reactivity for self-antigens such as DNA or insulin (Gitlin et al., 2016). Given our finding that cells with V_H1-81-containing BCRs dominate the PE-specific B cell repertoire in *Igh*^{b} mice, it is reasonable to postulate that V_H1-81 has a conformation that is conducive to acquisition of somatic mutation dependent self-reactivity and clonal deletion. More work must be done to confirm that this is the case and whether this mechanism accounts for other examples of declining B cell memory.

The fact that some antigen-specific swIg memory B cell populations are very stable (Crotty et al., 2003; Jones et al., 2015; Weisel et al., 2016) indicates that the instability is not a property of all swIg memory B cells. However, it has been reported that sheep red cell-and malaria antigen-specific swIg memory cells in mice (Dogan et al., 2009; Krishnamurty et al., 2016) and pertussis toxin-specific swIg memory cells in humans (Stenger et al., 2010) decline over time. Another particularly relevant example has been reported for human B cells specific for a conserved epitope on the influenza hemagglutinin stem region (Wheatley et al., 2015). These B cells utilize V_H1-69, depend primarily on a specific residue in the CDR-H2 for hemagglutinin binding, and declined dramatically over a 70-week period after booster vaccination, while non-stem-specific memory B cells were stable. These results provide another case where a B cell population with CDR-H2-focused BCRs generate unstable swIg memory B cells. Thus, swIg memory B cell decline may be more common than previously appreciated.

The different PE-specific immune responses in *Igh*^{b} but not *Igh*^{a} mice are consistent with the effects of Ig polymorphism reported for other B cell repertoires. About 50% of the antibodies specific for the hapten p-azophenylarsonate in the A/J strain mice contain an idiotypic epitope that is not present in p-azophenylarsonate antibodies produced by other strains (Pawlak et al., 1973), which is due to a polymorphic gene in the *Igh* locus (Siekevitz et al., 1982). Likewise, the capacity of certain mouse strains to produce thyroid stimulating hormone receptor antibodies maps to the *Igh* locus (Rapoport et al., 2010). Several studies indicate that humoral responses can also vary in people depending on the germline-encoded V_H segments that they express (Field et al., 2002; Raposo et al., 2014; Sakkas et al., 1987; Wheatley et al., 2015). A case of a single germline-encoded V segment controlling immunity has been reported for the human B cell response to the *Hemophilus influenza* type B (Hib) capsule (Feeney et al., 1996). Most people produce Hib capsule-specific antibodies

that contain a single IgL chain containing a V segment encoded by the *IGVKA2* gene. These antibodies bind with high affinity to the Hib capsule without somatic mutations. The importance of these antibodies is evidenced by the fact that some people have *IGVKA2* genes that cannot undergo productive rearrangement and these individuals are at high risk for contracting Hib infection. Together, the evidence is consistent with the possibility that the naïve Hib capsule-specific B cell population in humans can be dominated by cells with BCRs that bind to Hib mainly with the germline-encoded region of one Vκ chain. A similar concept applies to T cells as work from Harty and colleagues (Van Braeckel-Budimir et al., 2017) indicates that $CD8^+$ T cells in a malaria epitope-specific population bind to their ligand with the germline-encoded region of one T cell antigen receptor beta chain. These results suggest that antigen receptors that depend heavily on germline-encoded elements for antigen binding are a general feature of the T and B cell pre-immune repertoires for certain epitopes.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marc K. Jenkins (jenki002@umn.edu).

EXPERIMENT MODEL AND SUBJECT DETAILS

Mice were housed in a specific pathogen-free facility at the University of Minnesota, and experiments were conducted according to federal and institutional guidelines and with the approval of the University of Minnesota Institutional Animal Care and Use Committee. Six- to 12-week-old, male and female, age and sex-matched mice were used for all experiments.

B cell hybridomas, HEK 293T, and SP2/0-Ag14 cells were maintained in Dulbecco's Modification of Eagle's Medium (cellgro) supplemented with 10% Fetal Bovine Serum, L-Glutamine, HEPES buffer, Non Essential Amino Acids (NEAA), sodium pyruvate, penicillin/streptomycin, and gentamycin. For antibody production, B cell hybridomas were transferred to Hybridoma-SFM (Gibco) supplemented with L-Glutamine, penicillin/streptomycin, and gentamycin. All cultures were incubated in a humidified chamber at 10% $C0_2$ and 37°C. All of the cell lines were of female origin.

METHOD DETAILS

Animals and immunizations—B6 mice (C57BL/6NCr, Igh^b) and B6, CD45.1 mice (B6-Ly5.1/Cr, Igh^b) were purchased from Charles River/National Cancer Institute. BALB/c (BALB/cAnNCrl, Igh^a) mice and C.B-17 mice (C.BKa-Igh^b/IcrCr, Igh^b) were purchased from Charles River. BALB/c, CD45.1 mice (B6-Ly5.1/Cr, Igh^a) were purchased from Jackson Labs. Recombination-activating gene-deficient MD4 mice were bred in our facility using MD4 mice (C57BL/6-Tg(IghelMD4)4Ccg/J) (Goodnow et al., 1988) and Rag1 −/− mice (B6.129S7-Rag1tm1Mom/J) both purchased from Jackson Laboratories. Animals were injected subcutaneously in the base of the tail with 50 μl of CFA (Sigma) emulsion containing either 15 μg of APC and/or 15 μg PE (Prozyme). For adoptive transfer experiments, purified naïve B cells were prepared from the spleen and lymph nodes of

CD45.1^{+} congenic mice with a negative selection kit from Miltenyi, and 2×10^{7} cells were injected intravenously into recipient mice.

Hybridomas and recombinant antibodies—The ClonaCell®-HY kit was used to produce B cell hybridomas. Briefly, female B6 mice were immunized with PE as described above and then boosted two weeks later with an intravenous injection of PE. Four days later, the spleen cells were fused with SP2/0-Ag14 myeloma cells in the presence of polyethylene glycol and hybridomas selected and cloned in methyl cellulose containing hypoxanthine-aminopterin-thymidine. Four monoclonal antibody-secreting hybridomas, CL20, CL21, CL30, and CL33 were identified by screening hybridoma supernatants for PE-binding using an enzyme-linked immunosorbent assay (ELISA).

Recombinant versions of the CL30 and CL33 monoclonal antibodies were generated as described (von Boehmer et al., 2016). The *Ighv*, *Iglv*, or *Igkv* genes from each hybridoma were isolated using a first PCR performed with mixtures of forward primers specific for the *Ighv*, *Iglv*, or *Igkv* leader regions and reverse primers specific for the Ighg constant region. The products of this PCR, were subjected to a second PCR with mixtures of forward primers specific for sequences from the 5′ ends of the *Ighv*, *Iglv*, or *Igkv* genes and a nested reverse primer specific for the *Ighg* constant region. The DNA sequences of these products was determined and the results used to order gene blocks from IDT encoding the CL30 or CL33 IgH and Igκ chains (with the exception that the sequence encoding the CDL33 CDR-H2 was changed from IYPKSGNT to IYPRSGNT) or recombinant versions with CDR-H2 or CDR-H3 replacements. The assembled gene blocks contained 16 base pair overlapping regions on both ends for infusion cloning (Takara Bio) into the AgeI and SalI-linearized AbVec2.0-IGHG1 plasmid encoding the human Cγ1 constant region (Addgene - 80795) (Tiller et al., 2009). For the Igκ chains, two more codon-optimized gene blocks encoding the VJ segment of the respective chains of CL33 and CL30 were generated and cloned into the AgeI and BsiWI-linearized vector Abvec1.1-IGKC (Addgene - 80796) (Tiller et al., 2009). The gene blocks used for generating the recombinant antibodies are shown in the Table S12.

The expression vectors encoding the relevant IgH and Igκ segments for each B cell were co-transfected into HEK 293 cells, and the antibodies secreted into the culture supernatants were purified on protein G. Concentrations of human IgG in the purified supernatants were determined by sandwich ELISA. Briefly, 96-well plates were coated with 10 μg per ml goat anti-human IgG Fc gamma (Jackson Immunoresearch) in PBS and blocked with 1% BSA in PBS. Serial dilutions of each sample were added to the plates along with a purified human IgG standard. Plate-bound human Ig was detected by incubating the wells sequentially with biotin-conjugated anti-human Ig Fc gamma (eBioscience), streptavidin-conjugated horse radish peroxidase (Perkin Elmer), and ABTS Peroxidase Substrate (KPL).

PE-binding ELISAs were performed by coating of 96-well plates with 20 μg per ml PE in PBS and blocking with 1% BSA in PBS. Serial dilutions of the recombinant human IgG antibodies or purified mouse monoclonal antibodies starting at 10 μg per ml were added, and bound Ig was detected with biotin-conjugated anti-human Ig Fc gamma (eBioscience) and streptavidin-conjugated horse radish peroxidase (Perkin Elmer) or HRP-anti-mouse Ig heavy

and light chain antibody (Thermo Scientific), followed by ABTS Peroxidase Substrate (KPL). In some cases, the titer at half-maximal OD 405 was calculated for each sample.

Cell enrichment—Explanted lymph nodes and spleens were minced in collagenase and EDTA as previously described (Itano et al., 2003) and then passed through fine mesh, washed, and suspended in 0.2 ml of culture supernatant containing 24G2 antibody (ATCC, Manassas) plus 1% rat serum (Sigma) to block Fc receptors. Each sample was incubated with 1 μg PE or APC for 30 minutes at 4°C, washed with sorter buffer (PBS containing 0.1% NaN3 and 2% fetal bovine serum), and incubated with 25 μl anti-PE or anti-APC microbeads (Miltenyi) for 15 minutes at 4°C. Samples were then washed, suspended in 3 ml of sorter buffer, and passed over magnetized LS columns. The columns were washed 3 times to remove unlabeled cells. After the last wash, the columns were removed from the magnetic field, and bound cells were eluted in 5 ml of sorter buffer.

Flow Cytometry and Cell Counts—Antibodies used for flow cytometry were from eBioscience (San Diego) unless otherwise indicated. Cell suspensions were incubated with FITC-labeled anti-CD79b (BioLegend), PercP-Cy5.5-labeled anti-mouse IgM, Alexa Fluor 700-labeled anti-CD38, eFluor 450-labeled Gl-7, BV786-labeled anti-mouse IgD, BUV395-labeled anti-B220 (BD Biosciences), and either APC-eFluor780-labeled anti-CD90.2, F4/80, CD11c, and Gr-1 or BV711-labeled anti-CD4 (BD Biosciences), CD8 (BD Biosciences), F4/80 (BioLegend), CD11c (BioLegend), and Gr-1(BioLegend). In some samples a BV605-labeled anti-CD45.1 antibody was also included. The cells were then fixed in Cytofix/Cytoperm (BD Biosciences), washed with Perm Buffer (TONBO) and incubated with AF350-labeled anti-mouse IgH and IgL chain antibody (ThermoFisher). Fluorescent beads (Invitrogen) were used to calculate total numbers of live lymphocytes in spleen or lymph node suspensions. To obtain the total number of PE- or APC-binding cells in a sample, this value was multiplied by the frequency of cells binding either PE or APC. For naïve mice, only the PE- or APC-enriched fractions were used to obtain the total number of cells, as the majority of antigen-binding cells are present in this fraction (Pape et al., 2011). For day 6–50 after immunization, the number of antigen-binding cells in the fraction that flows through the enrichment columns was added to the numbers from the enriched fraction. Flow cytometry was performed on a 5-laser (355 nm, 405 nm, 488 nm, 561 nm, 640 nm) LSRFortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Ighv Sequencing—For sequencing of cell from the naïve repertoire, suspensions of spleen and lymph nodes from naïve BALB/c, C.B-17, or B6 mice were stained with PE, eFluor 450-labeled B220, and APC-eFluor780-labeled anti-CD90.2, F480, CD11c, and Gr-1 (non-B cell lineage). For sequencing of memory B cell repertoires, spleen and lymph node cells from PE/CFA immunized BALB/c or B6 mice were stained with PE, BV650-labeled B220, AF700-labeled anti-CD38, FITC-labeled GL-7 (BD Bioscience) and APC-eFluor780-labeled anti-CD90.2, F480, CD11c, and Gr-1 (non-B cell lineage). PE-binding cells were enriched with anti-PE microbeads as described above and non-B cell lineage negative, PE^+, $B220^+$ cells (for naïve repertoire) or non-B cell lineage negative, PE^+, $B220^+$ cells that were $CD38^+$, GL7-negative (memory) were sorted using a FACS Aria II (640, 405, 561 nm lasers)

into sorter buffer and pelleted. For hybridoma sequencing, cells growing in log phase were washed in PBS and pelleted. The cell pellets were then frozen at −80°.

Cell pellets were lysed in trizol reagent (Invitrogen) and RNA isolated by mixing 1:1 with 100% ethanol and applying to Zymo-Spin IIICG columns (Zymo Research). cDNA was generated using First-strand cDNA synthesis kit (Invitrogen) per manufacturers recommendation. *Ighv* alleles were amplified with Herculase II DNA polymerase (Agilent) using a common variable region primer msVHE (5′ – GGGAATTCGAGGTGCAGCTGCAGGAGTCTGG – 3′) (Seidl et al., 1997) and specific constant chain primers IgM reverse (5′ – GATACCCTGGATGACTTCAGTGTTG – 3′) or IgG reverse (5′ – CACACCGCTGGACAGGG – 3′). PCR products were separated on a 1% agarose gel and the 500 base pair fragment was gel extracted (Qiagen). Samples were cloned into StrataClone PCR cloning kit (Agilent) and plasmids sequenced. Variable regions were identified using IgBLAST software from the National Center for Biotechnology Information. Due to limited BALB/c *Ighv* sequence information, antibodies genes from BALB/c samples were identified as the closest homologous sequence found within the B6 database. CDR-H3 sequence identity was determined using IMGT sequence definitions (Lefranc et al., 2003).

PE-blocking experiments using anti-PE monoclonal antibody—The CL33 anti-PE hybridoma was produced in house from PE/CFA-injected B6 mice, and subjected to *Ighv* sequencing (see above). This analysis showed that CL33 used V_H1-81, D_H1-1, and J_H3 to encode its IgG2b heavy chain. The Fab form of the antibody was prepared using a kit (Thermo Scientific). In epitope blocking experiments, PE enrichment was performed as described above except that the PE was mixed with a 20-fold molar excess of CL33 Fab in sorter buffer, or sorter buffer alone, for 20 minutes before addition to cell suspensions.

QUANTIFICATION AND STATISTICAL ANAYSIS

Prism software (Graphpad) was used to calculate *p* values were determined by an unpaired two-tailed Student's t test. Differences between groups were considered significant for *p* values ≤ 0.05.

DATA AND SOFTWARE AVAILABILITY

All the *Ighv* gene nucleotide sequence data is deposited on Mendeley Data (https://data.mendeley.com) DOI: 10.17632/bb7k3g6vbg.2

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A population of antigen-specific naïve B cells mainly expresses a single V_H segment.
- This V_H confers high-affinity antigen binding that depends on an un-mutated CDR-H2.
- B cells with this V_H produced many plasmablasts and few germinal center cells.
- B cells with this V_H produced abundant stable IgM^+ and transient IgG^+ memory cells.

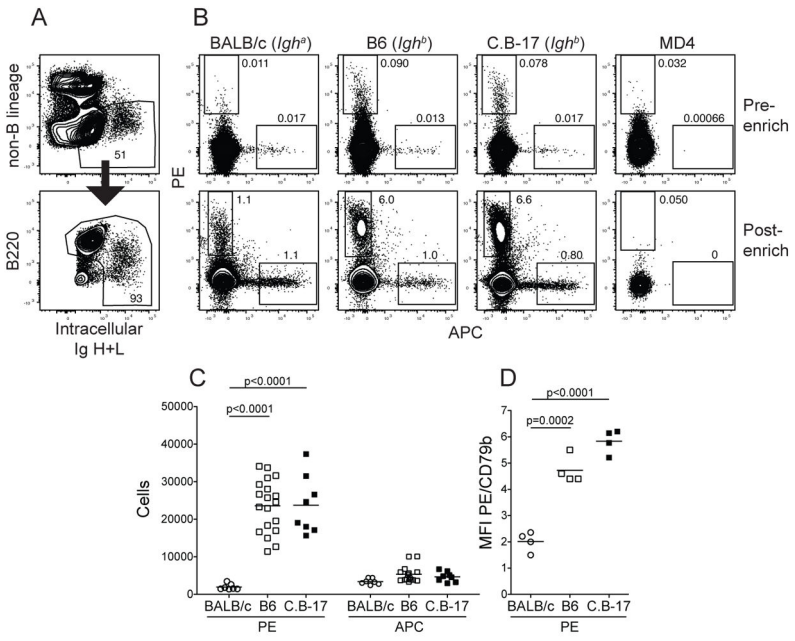

Figure 1. *Igh*^{a} and *Igh*^{b} mice have different numbers of naïve PE-specific B cells

(**A**) Contour plots showing gating strategy to identify non-B cell (CD4, CD8, CD11c, Gr1, and F480) lineage^{-} Ig^{+} (upper panel) B220^{+} (lower panel) B cells in a sample of spleen and lymph nodes cells enriched for PE- and APC-binding cells.

(**B**) Contour plots of PE versus APC staining of unenriched and PE- and APC-enriched B cells from the spleen and lymph nodes of naive mice of the indicated strains. The cells were pre-gated as in (**A**) above. The percentages of cells binding either antigen are indicated in the rectangular gates.

(**C**) Scatter plot showing the numbers of B cells binding PE versus APC in the spleen and lymph nodes of the indicated naïve mouse strains. Each value represents an individual mouse. The data is pooled from 4 experiments, and mean values are indicated by a horizontal bar. Significant *p* values from an unpaired T-test are shown for the comparison of mean values from BALB/c and B6, and BALB/c and C.B-17.

(**D**) The relative amount of PE binding on naïve PE^{+} B cells from PE-enriched samples of spleen and lymph nodes from the indicated mouse strains. Each point represents the mean fluorescence intensity (MFI) of cell surface PE divided by the MFI of cell surface CD79b from an individual mouse. The data is pooled from 2 experiments and mean values are indicated by a horizontal bar. Significant *p* values from an unpaired T-test are shown for the comparison of mean values from BALB/c and B6, and BALB/c and C.B-17.

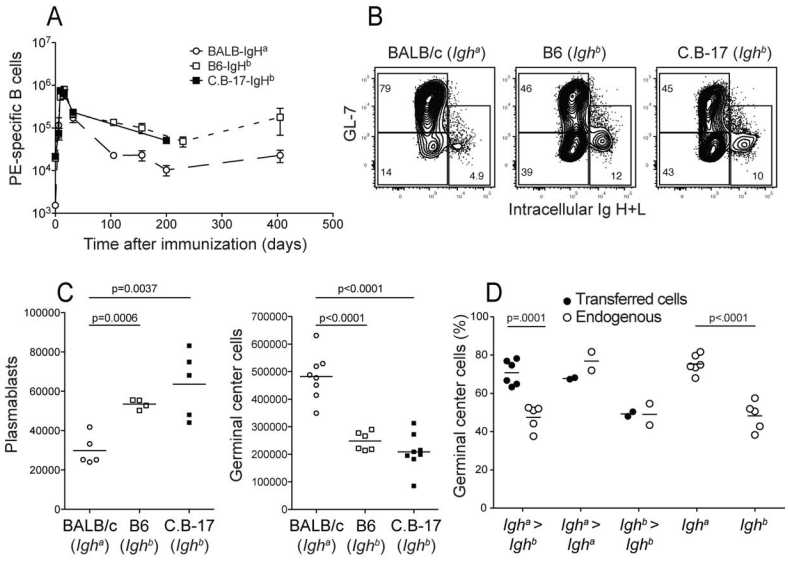

Figure 2. PE-specific B cell expansion and differentiation dynamics in *Igh*^{a} and *Igh*^{b} mice following immunization

(**A**) Combined data from 5 separate experiments showing the numbers of PE-specific B cells in the spleen and lymph nodes of *Igh*^{a} or *Igh*^{b} mice at the indicated times after immunization with PE in CFA. Mean values ± SEM are shown for each time point (n=2–8).

(**B**) Contour plots depict PE-binding B cells in PE-enriched spleen and lymph node samples from the indicated mice primed with PE in CFA 9 days before analysis. The rectangular gates on each plot shows the frequency of GL7$^+$ intracellular Ig^{lo} germinal center (GC) cells, GL7$^-$ intracellular Ig^{hi} plasmablasts (PB), or the GL7$^-$ intracellular Ig^{lo} memory cells.

(**C**) The total number of PE-specific plasmablasts on days 9–13 (left plot), pooled from 2 separate experiments, or germinal center cells on days 9–16 (right plot), pooled from 3 separate experiments, in spleen and lymph node samples from the indicated mouse strains after immunization with PE in CFA. Each point represents the frequency of each subset (gated as in **B** above) multiplied by the total number of PE-binding cells for an individual mouse. Mean values are indicated by a horizontal bar. Significant *p* values from an unpaired T-test are shown for the comparison of mean values from BALB/c and B6, and BALB/c and C.B-17 mice.

(**D**) Combined data from two experiments showing the percentage of PE-binding germinal center cells enriched from the spleen and lymph nodes of mice that were immunized with PE in CFA 17 days earlier. Some of the mice received naïve B cells from a CD45.1$^+$ strain. The mice were C.B-17 (CD45.1$^-$ *Igh*^{b}) mice that received B cells from BALB/c (CD45.1$^+$ *Igh*^{a}) mice (*Igh*^{a}>*Igh*^{b}), BALB/c (CD45.1$^-$ *Igh*^{a}) mice that received B cells from BALB/c (CD45.1$^+$ *Igh*^{a}) mice (*Igh*^{a}>*Iga*^{a}), B6 (CD45.1$^-$*Igh*^{b}) mice that received B cells from B6 (CD45.1$^+$ *Igh*^{b}) mice (*Igh*^{b}>*Igh*^{b}), intact BALB/c (*Igh*^{a}) or intact C.B-17 (*Igh*^{b}) mice. Each point represents the frequency of transferred (CD45.1$^+$) or endogenous (CD45.1$^-$) germinal center B cells (gated as in **B** above) in the PE-binding population of an individual mouse (n=2–6). Mean values are indicated by a horizontal bar. Significant *p* values from an unpaired T-test are shown for comparisons of mean values for transferred and endogenous populations, and endogenous populations in intact *Igh*^{a} and *Igh*^{b} mice.

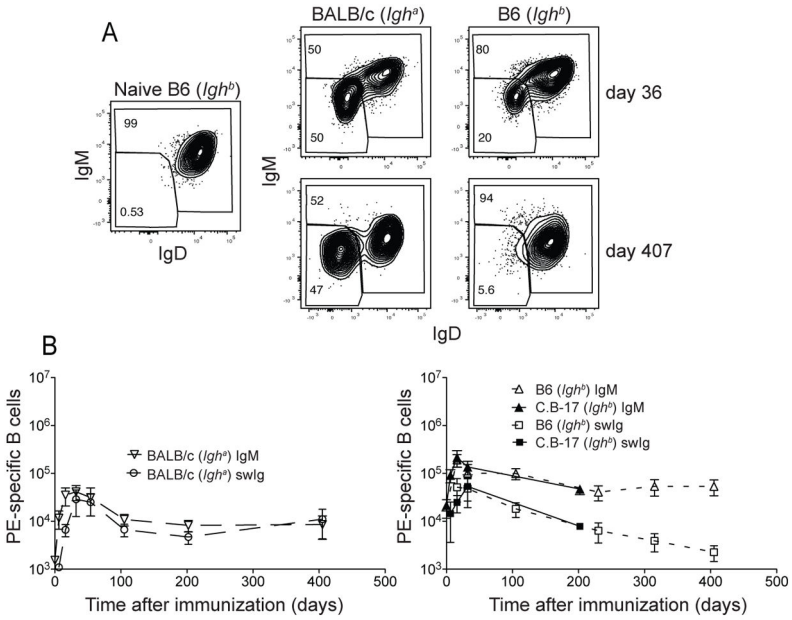

Figure 3. PE-specific memory B-cell kinetics and affinity maturation following immunization

(**A**) Contour plots showing the frequency of PE-binding IgM (IgM^+, IgD^+) versus swIg (IgM^-, IgD^-) cells in the pool of $GL7^-$ intracellular Ig^{lo} memory cells (gated as in Figure 2B) in PE-enriched samples from the indicated mice at the indicated times after injection of PE in CFA.

(**B**) Combined data from 5 separate experiments showing the total number of PE-specific IgM or swIg memory cells in the spleen and lymph nodes of *Igh*^{a} mice (left plot) or *Igh*^{b} mice (right plot) at the indicated times after immunization with PE in CFA. Mean values ± SD are shown for all time points (n=3–6).

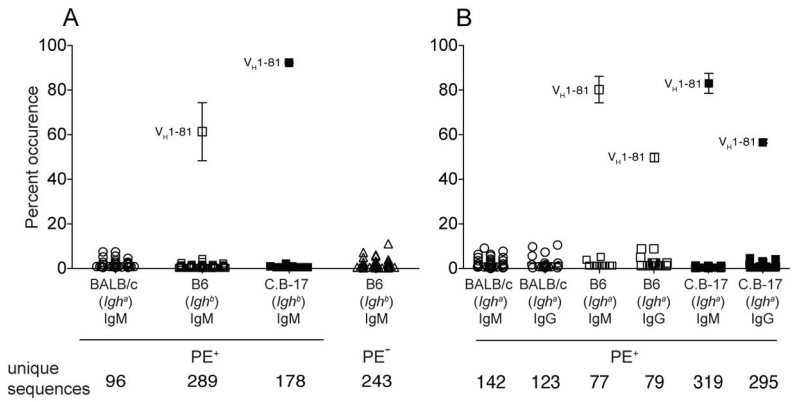

Figure 4. V_H usage by PE-specific B cells

(**A–B**) *Ighv* genes were PCR amplified, cloned and sequenced from 1000–20,000 purified CD38$^+$, GL7$^-$, PE-binding B cells per mouse that were sorted from PE-enriched samples (gated as in Figure 1A and B). Each value represents the frequency of an individual *Ighv* gene in the (**A**) naïve IgM or (**B**) memory IgM and IgG (day 30–168 after PE in CFA) repertoires of unique clones obtained from 2–3 mice per strain. PE-negative B cells were analyzed in (**A**) as a control for PCR primer bias. The total number of unique clones examined per strain is indicated below each graph. For the V_H1-81 gene, the mean values ± SEM are shown. For all other genes, only the mean is shown. See also Table S1–S4 (naïve) and Table S5–S10 (memory) for the sequence data from each sample.

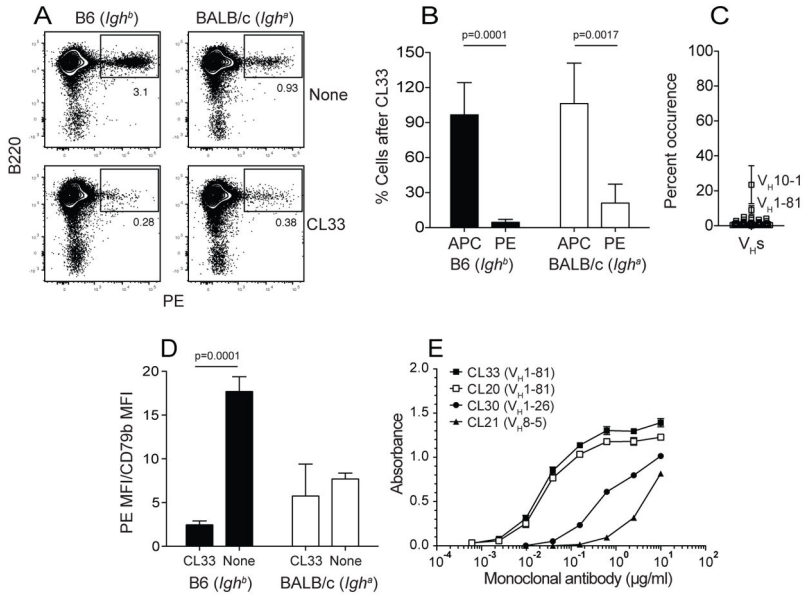

Figure 5. Most PE-specific B cells bind to a single epitope

(**A**) Contour plots of naïve *Igh*^{b} or *Igh*^{a} spleen and lymph node samples that were divided in half and mixed with PE in the presence (lower plots) or absence (upper plots) of a V_H1-81-containing anti-PE monoclonal antibody Fab (CL33) prior to PE-enrichment. The percentages of cells binding PE are indicated in the rectangular gates.

(**B**) The fraction of the PE$^+$ or APC$^+$ B cells that were detected after CL33 Fab treatment was calculated by dividing the number of PE$^+$ or APC$^+$ B cells in the CL33-treated portion by the number of PE$^+$ or APC$^+$ B cells in the untreated portion of an individual sample. Mean percentages ± SD are shown from the indicated strains (n=4–5 mice per group from two experiments). Significant *p* values from an unpaired T-test are shown.

(**C**) V_H usage analysis (as described in Figure 4) of PE-specific B cells from B6 (*Igh*^{b}) mice that bound PE in the presence of CL33 monoclonal antibody. The number of unique clones obtained from 3 sorted samples each containing 3 pooled mice/sample is indicated below the graph. For the V_H1-81 and the V_H10-1 gene, the mean values ± SEM are shown. For all other genes, only the mean is shown. Also see Table S11 for the sequence data.

(**D**) Relative PE affinities (calculated as in Figure 1D) of PE-specific naïve B cells from *Igh*^{b} or *Igh*^{a} mice that bound PE in the presence or absence of CL33 Fab. Mean values ± SD are shown (n=4). Significant *p* values from an unpaired T-test are shown.

(**E**) Binding of the indicated amounts of four monoclonal antibodies offered to PE-coated coated plates determined by ELISA. Mean absorbance values ± range are shown for duplicate determinations of CL33 and CL20, and single determinations of CL30 and CL21.

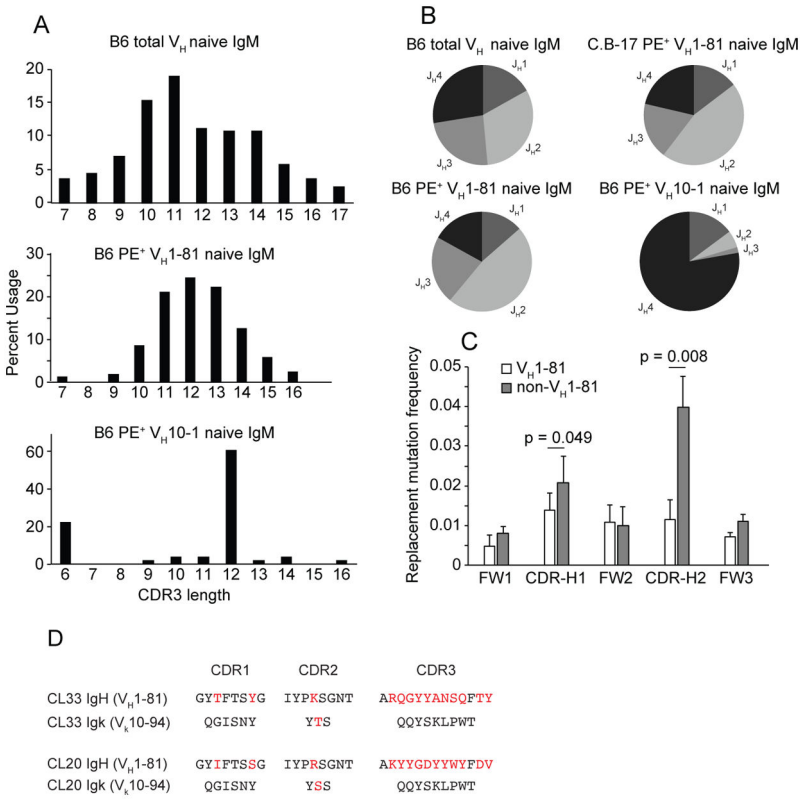

Figure 6. Naïve V_H1-81-expressing PE-binding B cells have diverse CDR3s

(**A**) CDR3s were identified as described by IMGT (Lefranc et al., 2003) from the *Ighv* gene sequences we obtained from naïve B6 (*Igh*^{b}) mice in Tables S2, S4, and S11. The percentage of CDR3s of specified lengths were plotted as histograms. The top plot shows PE-negative IgM B cells and contains 243 unique sequences obtained from 3 B6 mice. The middle plot shows PE$^+$ V_H1-81$^+$ naïve IgM B cells and contains 177 unique sequences obtained from 3 B6 mice. The bottom plot shows PE$^+$ V_H10-1$^+$ naïve IgM B cells and contains 54 unique sequences obtained from 10 B6 mice.

(**B**) The frequency of J_H usage by PE-negative IgM B cells from B6 mice (upper left), PE$^+$ V_H1-81$^+$ naïve IgM B cells from C.B-17 mice (upper right), PE$^+$ V_H1-81$^+$ naïve IgM B cells from B6 mice (lower left), and PE$^+$ V_H10-1$^+$ naïve IgM B cells from B6 mice (lower right). See also Tables S2, S3, S4, and S11.

(**C**) The frequency of mutations resulting in an amino acid changes in the indicated V_H segments were calculated from the sequences shown in Tables S8 and S10. Error bars represent the standard deviation. Significant *p* values from an unpaired T-test are shown.

(**D**) Amino acid sequences of the IgH and Igκ chains of the indicated regions of the CL20 and CL33 monoclonal antibodies. Differences are indicated in red font.

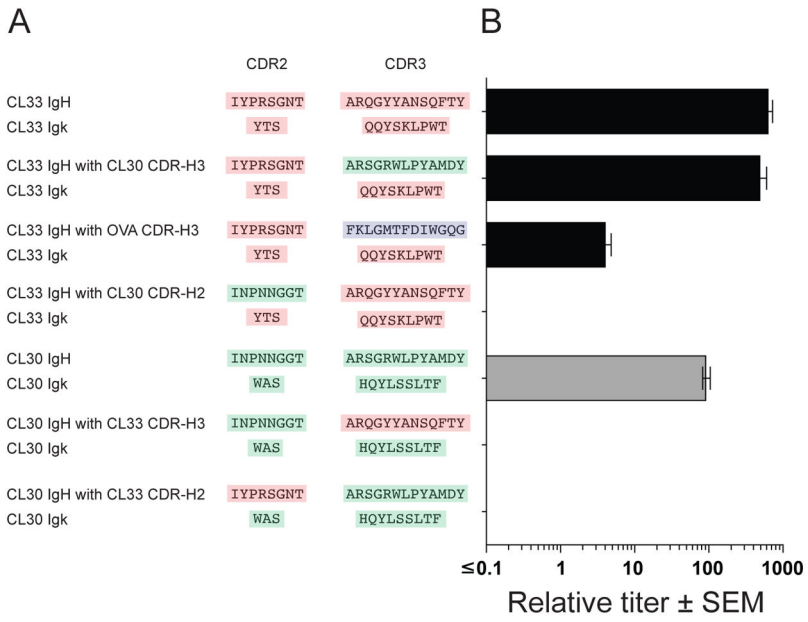

Figure 7. A V_H1-81-containing monoclonal antibody is tolerant of changes to its CDR-H3

(**A**) Sequences of the CDR2 and CDR3s of recombinant antibodies. Segments derived from CL33 are shown in red, from CL30 in green, or from an ovalbumin-specific antibody in purple. (**B**) Purified recombinant antibodies were serially diluted onto PE-coated plates at starting Ig concentrations of 10 μg/ml. Mean values ± SD on the X-axis indicate PE binding titer for each antibody determined by ELISA (n=3–5 per antibody).