We have recently demonstrated that a novel somatically mutated B220<sup>−</sup> memory B cell subset rapidly dominates the secondary immune response to (4-hydroxy-3-nitrophenyl) acetyl (NP). Upon adoptive transfer with Ag, B220<sup>−</sup>NP<sup>+</sup> memory B cells produce large numbers of B220<sup>−</sup>NP<sup>+</sup> B cells that can rapidly differentiate into plasma cells. Therefore, it is not clear whether the novel B220<sup>−</sup> memory compartment is a consequence of secondary Ag challenge or whether it develops as a stable memory subset after initial Ag challenge. In this study, we demonstrate the gradual emergence of B220<sup>−</sup>NP<sup>+</sup> B cells in the spleen to maximal numbers 3 wk after initial Ag exposure. Like their B220<sup>+</sup> counterparts, the B220<sup>−</sup> B cells initially appear unmutated at days 5–7; however, the majority rapidly accumulate affinity increasing mutations by days 9–14 of the primary immune response. More extensive cell surface phenotype (GL7<sup>−</sup>BLA-1<sup>−</sup>CD24<sup>−</sup>CD43<sup>+</sup>) argues strongly against germinal center localization and direct analysis in situ places a cohort of B220<sup>−</sup>CD11b<sup>+</sup>NP<sup>+</sup> B cells in the red pulp of the spleen and not in the MZs. These data provide direct evidence for the development of B220<sup>−</sup> memory B cells as a unique cellular consequence of primary Ag exposure. The cellular dynamics and molecular attributes of these unique memory B cells suggest they are distinct cellular products of the germinal center reaction in the primary response and are maintained long-term in the spleen and bone marrow. The Journal of Immunology, 2001, 167: 1393–1405.

B cell memory is characterized by the rapid appearance of high-affinity Abs in response to secondary Ag challenge. This accelerated humoral recall response is due to the expansion of affinity-matured memory response precursors and their rapid differentiation into plasma cells (1). We have recently identified two distinct subsets of isotype-switched (IgM<sup>−</sup>IgD<sup>−</sup>IgG<sup>+</sup>) and somatically mutated memory B cells that do not secrete Ab, but can give rise to plasma cells upon adoptive transfer with Ag (2). One of these subsets expresses high levels of the B lineage marker B220 (the B cell isoform of CD45R; RA3-6B2 binding) and CD19. These B220<sup>−</sup> memory B cells expand to peak levels by day 4 following secondary Ag challenge and persist in the spleen for at least 6 wk. Although the second memory subset does express CD45R (30-F11 binding), they do not express the B cell isoform B220 or CD19. These novel B220<sup>−</sup> memory responders emerge in the spleen with kinetics similar to those of the B220<sup>+</sup> subset, but persist long term at much higher frequencies. This unique B220<sup>−</sup> memory subset also comprises >95% of the Ag-specific B cells in the bone marrow (BM). Thus, a novel B220<sup>−</sup> memory B cell subset emerges following secondary challenge as a major cellular component of the Ag-specific memory B cell response and contributes significantly to the rapid appearance of high-affinity Ab.

Although the B220<sup>−</sup> subset clearly emerges upon secondary challenge, it is not known how this novel subset of B cell memory develops. Although our previous work provides some indication for both subsets of specific memory responders before secondary Ag challenge, cell numbers are very low at this point and are not significantly greater than the adjuvant-only controls (2). In addition, adoptive transfer experiments in this study demonstrate a hierarchical parent-progeny relationship in which B220<sup>−</sup> memory B cells produce nonsecreting B220<sup>−</sup> B cell intermediates that then give rise to plasma cells. This pattern suggests that the B220<sup>−</sup> population seen to expand upon secondary challenge could actually be a product of the B220<sup>−</sup> memory B cell subset. Thus, questions regarding the development of these unique B220<sup>−</sup> memory B cells cannot be answered simply by extrapolation from our previous findings on memory responders and their progeny.

Since both memory B cell subsets express somatically mutated Ig, it is likely that their precursors originate in the germinal center (GC) reaction during the primary response (3, 4). Although there have been reports of somatic mutation in the absence of GC formation in lymphotoxin α<sup>−/−</sup> (5) and Lyn-deficient animals (6), somatic mutation in both cases was reported only after tertiary immunization, and GC have subsequently been observed in the mesenteric lymph nodes of the lymphotoxin α<sup>−/−</sup> mice (7). The greater weight of evidence supports the GC microenvironment as the site of somatic mutation and affinity-based selection in the development of B cell memory (8–14). Therefore, we propose that the B220<sup>−</sup> memory B cells either 1) develop in the GC during the primary response, 2) are the progeny of primary response GC B cells, or 3) are the progeny of B220<sup>−</sup> memory B cells and arise only as a consequence of Ag recall.
To address this issue directly, we used the Th cell-dependent response to the hapten 4-hydroxy-3-nitrophenyl acetyl (NP) in C57BL/6 mice (15). The primary response to NP in these mice is dominated by B cells that express the V\(\gamma\)\(4\)\(8\)\(2\) H chain and the V\(\gamma\)A1 L chain genes (16–19). NP\(^+\) GC B cells reach maximum numbers by day 7 of the primary splenic response and persist at these levels for at least 3 wk in the spleen (16). Somatic hypermutation has been observed as early as day 6 after priming and progressively increases for the duration of the GC reaction (16, 20). GC B cells bind high levels of peanut agglutinin (PNA) (21) and express B220\(^+\) NP\(^+\) cells emerge more gradually to peak levels by day 21. Both B220\(^+\) NP\(^+\) B cells and B220\(^-\) NP\(^+\) B cells persist in the spleen at similar numbers for at least 8 wk postpriming. The kinetics of appearance and pattern of somatic mutation in the B220\(^-\) B cell population are consistent with these cells being an affinity-selected cellular product of the GC reaction. The B220\(^-\) subset appears abruptly and dominates the BM NP\(^+\) B cell compartment at day 7 after priming, but does not reach peak cell numbers until day 21. The major division of B220\(^-\) B cells previously reported in the memory response (IgG\(\mathrm{C} \)CD11b\(^+\) and IgE\(\mathrm{C} \)) also develops after initial priming. Cell surface phenotype and localization in situ indicate that the B220\(^-\) B cells are not found within the GC and are most likely the recirculating cellular outcomes of the GC reaction. Finally, the B220\(^-\) subset lacks key signaling molecules (CD19 and CD22), which may indicate unique requirements for reactivation of these cells upon Ag recall. Thus, the B220\(^-\) population initially develops in the primary response, undergoing mutation and affinity-driven selection in the GC, and persists as a major component of the post-GC memory B cell compartment.

Materials and Methods

Isolation of NP-specific B cells

Female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) at 6–10 wk of age were immunized i.p. with 400 μg NP-keyhole limpet hemocyanin (KLH) in Ribi adjuvant (Corixa, Hamilton, MT). For analysis of the memory response, mice were reimmunized with the same dose and route of Ag in adjuvant 8 wk postprimary immunization. Splenectomized and BM was aspirated from both femurs at various time points following primary immunization. To address this issue directly, we used the Th cell-dependent response to the hapten 4-hydroxy-3-nitrophenyl acetyl (NP) in C57BL/6 mice (15). The primary response to NP in these mice is dominated by B cells that express the V\(\gamma\)\(4\)\(8\)\(2\) H chain and the V\(\gamma\)A1 L chain genes (16–19). NP\(^+\) GC B cells reach maximum numbers by day 7 of the primary splenic response and persist at these levels for at least 3 wk in the spleen (16). Somatic hypermutation has been observed as early as day 6 after priming and progressively increases for the duration of the GC reaction (16, 20). GC B cells bind high levels of peanut agglutinin (PNA) (21) and express B220\(^+\) NP\(^+\) cells emerge more gradually to peak levels by day 21. Both B220\(^+\) NP\(^+\) B cells and B220\(^-\) NP\(^+\) B cells persist in the spleen at similar numbers for at least 8 wk postpriming. The kinetics of appearance and pattern of somatic mutation in the B220\(^-\) B cell population are consistent with these cells being an affinity-selected cellular product of the GC reaction. The B220\(^-\) subset appears abruptly and dominates the BM NP\(^+\) B cell compartment at day 7 after priming, but does not reach peak cell numbers until day 21. The major division of B220\(^-\) B cells previously reported in the memory response (IgG\(\mathrm{C} \)CD11b\(^+\) and IgE\(\mathrm{C} \)) also develops after initial priming. Cell surface phenotype and localization in situ indicate that the B220\(^-\) B cells are not found within the GC and are most likely the recirculating cellular outcomes of the GC reaction. Finally, the B220\(^-\) subset lacks key signaling molecules (CD19 and CD22), which may indicate unique requirements for reactivation of these cells upon Ag recall. Thus, the B220\(^-\) population initially develops in the primary response, undergoing mutation and affinity-driven selection in the GC, and persists as a major component of the post-GC memory B cell compartment.

CD138\(^+\) plasma cells reach peak numbers by day 5 and B220\(^+\) NP\(^+\) GC B cells reach peak numbers by day 7. The B220\(^+\) NP\(^+\) B cells emerge more gradually to peak levels by day 21. Both B220\(^+\) NP\(^+\) B cells and B220\(^-\) NP\(^+\) B cells persist in the spleen at similar numbers for at least 8 wk postpriming. The kinetics of appearance and pattern of somatic mutation in the B220\(^-\) B cell population are consistent with these cells being an affinity-selected cellular product of the GC reaction. The B220\(^-\) subset appears abruptly and dominates the BM NP\(^+\) B cell compartment at day 7 after priming, but does not reach peak cell numbers until day 21. The major division of B220\(^-\) B cells previously reported in the memory response (IgG\(\mathrm{C} \)CD11b\(^+\) and IgE\(\mathrm{C} \)) also develops after initial priming. Cell surface phenotype and localization in situ indicate that the B220\(^-\) B cells are not found within the GC and are most likely the recirculating cellular outcomes of the GC reaction. Finally, the B220\(^-\) subset lacks key signaling molecules (CD19 and CD22), which may indicate unique requirements for reactivation of these cells upon Ag recall. Thus, the B220\(^-\) population initially develops in the primary response, undergoing mutation and affinity-driven selection in the GC, and persists as a major component of the post-GC memory B cell compartment.

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DNA sequencing. To screen second-round PCR products for positives, 5 μl of each reaction was run on a 1.5% agarose gel. Samples that yield a single band of the correct size were run over a CL-6B Sepharose column (Pharmacia, Piscataway, NJ) to separate PCR products from primers and were then directly sequenced as described previously (2). Briefly, 4 μl of each PCR product was mixed with 4 μl of Dye Terminator Ready Reaction Mix (Perkin-Elmer, Norwalk, CT) and primer. Primers used and volumes are as follows: for L chain amplification, 1.6 pmol of LAM.seq (5'-GGCT TTCTCGGACCTCCGA-3') and LAM.rev.(antisense) (5'-GCTGCTGCTAGTGAAGGCT-3'). Each set of PCR cycles began with a 95°C incubation for 5 min followed by 40 cycles of 95°C for 15 s, 50°C for 45 s, and 72°C for 90 s and ends with a 5-min 72°C incubation. For every 10 PCR, two negative controls (wells containing cDNA reaction mix into which no cells were sorted) were processed along side experimental samples through both rounds of PCR to control for contamination during sample processing.

In situ immunofluorescence

Splenectomy from mice 14 days postprimary immunization were snap frozen in OCT embedding compound (Miles Labs, Elkhart, IN) and stored at ~80°C until use. Six-micrometer-thick sections were cut using a cryostat microtome (Leica, Deerfield, IL) and placed onto gelatin-coated slides, air dried, and stored at 4°C until use, as previously described (30). Sections were rehydrated with PBS (pH 7.4) for 5 min before blocking for 30 min at room temperature in blocking buffer (PBS containing 10% FCS and 10% (w/v) skim milk powder). Sections
were then stained with APC-conjugated NP in blocking buffer overnight at room temperature. After washing with PBS, sections were blocked for 30 min at room temperature in blocking buffer containing 50% (v/v) anti-FcR (2.4G2 hybridoma supernatant). Alternatively, sections to be stained with biotin reagents were blocked with blocking buffer containing 2 μM biotin (Pierce, Rockford, IL) for 30 min at room temperature, rinsed, and blocked with blocking buffer containing 2 mM biotin (Pierce, Rockford, IL) for 30 min. Sections were then stained with combinations of the following Abs, as indicated in Fig. 6, for 1 h at room temperature: TR-11.26 (anti-IgD), FITC-RA3.6B2 (anti-B220), FITC/biotin-M1/70 (anti-CD11b), FITC-R35-72 (anti-IgE), and biotin-281.2 (anti-CD138). Biotin-stained sections were then stained with neutravidin-rhodamine (Molecular Probes, Eugene, OR) for 1 h at room temperature. Sections were washed in PBS and mounted in VectorShield (Vector Laboratories). Data were acquired using a Zeiss Axioplan LSM 410 microscope system (Zeiss, Oberkochen, Germany) with each signal collected serially in the first detector using LSM 3.95 software and ×10 or ×40 objectives. The three separate images were optimized for signal-to-noise, colorized, and reassembled using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**Results**

*Development of an Ag-specific B cell response in the spleen*

Using flow cytometry, we can directly quantitate the emergence of Ag-specific B cells in the spleens of mice immunized i.p. with NP-KLH in Ribi adjuvant. Our strategy focuses on PI<sup>−</sup>, CD4<sup>−</sup>, and CD8<sup>−</sup>, F4/80<sup>−</sup> cells (Fig. 1A, left panel) that bind NP and have down-regulated IgM (data not shown) and IgD upon activation in vivo (Fig. 1A, upper left quadrant of NP/IgD plots; an NP<sup>+</sup>IgD<sup>−</sup> population also appears that is not further characterized in this study). By day 3 postprimary immunization, the NP-specific B cell population emerges significantly above baseline levels of day 0 (p < 0.001; one-way ANOVA) and KLH alone in adjuvant.

![Image](https://example.com/image1.png)

**FIGURE 1.** The emergence of three subsets of Ag-specific B cells in the spleen during the primary response. Female C57BL/6 mice immunized i.p. with 400 μg of NP-KLH in Ribi adjuvant were sacrificed at various times postimmunization and spleens were harvested and labeled for flow cytometry. **A.** The splenic NP-binding B cell population. PI-positive events (dead cells), T cells, and macrophages (as detected by Cy5-PE labeled anti-CD4, CD8, and F4/80) were excluded from further analysis and light scatter gates were broad so as not to exclude large B cell blasts. Remaining cells were analyzed for their expression of IgD (TR-11.26) and the ability to bind NP (APC-NP); the upper left quadrant of each plot indicates the level of fluorescence considered to be NP<sup>+</sup>IgD<sup>+</sup>. Cellular dynamics of the total NP-binding IgD<sup>+</sup>B cell population. Total numbers of NP-binding IgD<sup>+</sup>B cells in the spleen were calculated using frequencies obtained via flow cytometry and total splenic cell numbers obtained upon sacrifice of the animal. Each time point is composed of at least three animals with the means ± SEM displayed. **C.** Three subsets of Ag-specific B cells. The activated, Ag-specific B cell population (PI<sup>−</sup>, CD4<sup>−</sup>, CD8<sup>−</sup>, F4/80<sup>−</sup>, IgD<sup>−</sup>, NP<sup>+</sup>) was analyzed for the expression of CD138 (PE-281.2) and B220 (FITC-RA3-6B2) at the indicated days of the response. **D.** Cellular dynamics of the three NP-binding B cell subsets. Numbers of NP-specific B cells of the indicated phenotypes were calculated as in **B** at the indicated days of the response. Each time point represents the mean ± SEM of at least three animals.
controls (p < 0.05; Fig. 1B). An initial plateau in cell numbers is reached by day 7 (0.7% of total spleen cells) that is maintained through day 14. A second increase in the number of NP IgD- cells occurs from days 14 to 21 (p < 0.01; 1.4% of total spleen at day 21). The number of Ag-specific B cells markedly declined over the succeeding week (90% decline in NP+ cells from day 21 to day 28; 0.2% of total spleen at day 28) to levels that are maintained as a quiescent memory population for at least 8 wk (0.1% of total spleen; no change from days 28 to 56, p > 0.05). The number of NP+ cells detected 56 days postprimary immunization in this study was greater than previously reported (2). This is due to the use of an NP-APC reagent with an 8-fold reduced ratio of NP:APC that is capable of detecting a greater number of NP+ B cells without an increase in background staining. Thus, two waves of cellular expansion occur in the spleen during the first 3 wk of the primary response, with a quiescent memory population established by day 28.

### Induction and maintenance of a B220- memory B cell population

Expression of the cell surface markers B220 and CD138 divides the NP+ IgD- B cell population into three cellular subsets (Fig. 1, C and D). NP-specific Ab-secreting cells express intermediate levels of B220 and high levels of CD138 (2, 16, 27). This CD138- population emerges rapidly and reaches maximal numbers by the fifth day of the response (Fig. 1, C and D). There is a significant decrease in CD138- B cells by day 14 (p < 0.05) and a return to baseline levels by day 28. NP-specific GC B cells express high levels of B220 and do not express CD138 (2, 16, 27). As predicted from kinetic studies of the GC reaction (20, 31), this B220- population reaches peak cell numbers by day 7 and is maintained through day 21 (Fig. 1D). After a significant decline from days 21 to 28 (p < 0.05), the B220- population persists for at least 8 wk (no change from days 28 to 56; p > 0.05) as a post-GC B220- memory B cell population.

An NP-specific B220-CD138- B cell compartment clearly emerges during the primary response, with kinetics that are distinct from either the CD138+ or B220+ subsets (Fig. 1, C and D). By the end of the first week of the response, this B220- population accounts for nearly 50% of all NP-specific cells in the spleen (Fig. 1D). The second increase in the number of NP-specific B cells between days 14 and 21 is attributed to this B220- population, accounting for 85% of all NP-specific cells in the spleen by day 21. Similar to the other populations, there was a decline in the B220- population from days 21 to 28 to levels that are maintained for at least 8 wk (no change from days 28 to 56; p > 0.05). Although the B220- subset dominates the quiescent memory compartment following secondary immunization (2), the B220+ and B220- populations comprise similar proportions of the quiescent memory compartment in the spleen 8 wk postprimary immunization (~5:4:1 proportion of B220+ :B220- :CD138-). Thus, the B220+ Ag-specific memory B cell population develops in the primary response and is maintained as a major component of B cell memory.

### The B220- population expresses mutated Ig L chain genes

To evaluate whether B220- memory B cell development involves a GC phase, we assayed for the presence and prevalence of somatic hypermutation in single NP-specific B cells. The vast majority (>80%) of NP-specific responders in each subset express the L chain (data not shown). Individual NP-specific B cells from each subset were sorted for cDNA synthesis, L chain-specific RT-PCR, and DNA sequence analysis (representative sequences shown in Fig. 2, A–C; sequences are grouped by subset within the first (Fig. 2A) or second week (Fig. 2B) of the primary response or first week of the memory response (Fig. 2C)). As expected, few cells from any subset are mutated during the first week of the primary response (27%, n = 87; Fig. 2, A and D). The B220- subset was seen to emerge initially unmutated, possessing somewhat fewer total mutations (0.05%), mutations per mutated sequence (1.0), and replacement mutations (0%) than either the CD138+ or B220+ subsets during the first week of the response (Fig. 2D).

During the second week of the response, an increase in the frequency of mutated cells (53%, n = 75; Fig. 2, B and E) can be seen across all subsets. The B220- subset is now seen to be mutated to a similar level as the CD138+ and B220+ subsets (2.6 mutations per mutated sequence vs 2.3 and 2.1, respectively). Most notably, the percentage of replacement mutations in complementarity-determining region (CDR) 1 and 2 is similarly high across all subsets (range, 82–90%), a broad indication of Ag-driven selection (17). CD138+ memory responders were also sequenced (Fig. 2C), as we have shown that B220- memory cells can give rise to CD138+ Ab-secreting cells upon adoptive transfer (2). These memory responders serve as an indication of the level and pattern of mutation expected following affinity maturation in vivo. Although the frequency of mutation is greater than that seen during the second week of the primary response, the pattern of mutation and the percentage of replacement mutations in these memory responders is similar to that of all subsets in the primary response (Fig. 2, C and E). Thus, while the B220- cells initially emerge unmutated, they develop a frequency and pattern of mutations that is similar to the CD138+ and B220+ subsets as well as the CD138+ memory responders, providing evidence for a GC phase in their development.

### The B220- population expresses mutated Ig H chain genes

Although mutation is indicative of B cell memory and GC experience, affinity maturation offers the more reliable indicator. A mutation resulting in a tryptophan to leucine change at position 33 of CDR1 in the V\textsubscript{H}186.2 H chain gene confers a 10-fold increase in affinity for NP (32) and can serve as a marker of high-affinity NP-specific B cells. As observed in the L chain, few H chain mutations are observed among all three subsets during the first week of the response and the position 33 change is absent (Fig. 3, A and D). During the second week of the response, all three subsets are observed to accumulate extensive mutation in their H chain loci (Fig. 3, B and E). The B220- subset has a greater number of mutations per mutated sequence than either the CD138+ or B220+ subsets (6.4 vs 2.7 and 5.2, respectively; Fig. 3E). The majority of CD138+ and B220- cells possess the affinity-enhancing Trp to Leu mutation (75 and 68%, respectively; Fig. 3E), implying Ag-driven selection. Although the B220- subset has fewer mutations per mutated sequence than the CD138+ memory cells (6.4 vs 12; Fig. 3E), both subsets are similar in the percentage of replacement mutations and position 33 change. Thus, the B220- subset develops mutated H chain loci with evidence for Ag-driven selection, which is a molecular hallmark of GC experience and B cell memory.

Highly mutated B220- sequences do not represent non-V\textsubscript{H}186.2 J558 family member H chain genes, as a comparison of our total data set (n = 139) with the germline sequences of all known J558 family members (n = 67; kindly supplied by G. Kelsoe, Immunology Department, Duke University Medical Center, Durham, NC) revealed that the vast majority (99%) are more consistent with being mutated V\textsubscript{H}186.2 genes than other J558 family members (non-V\textsubscript{H}186.2 sequences were omitted from subsequent analyses). The frequency of obtaining a V\textsubscript{H}186.2-specific PCR product was consistently 50–65% of the \Lambda L chain frequency on a per cell basis, even for the CD138+ memory responders (65% \Lambda vs 28% V\textsubscript{H}186.2), as it is possible that non-V\textsubscript{H}186.2 genes are
FIGURE 2. Each of the three subsets of NP-specific B cell populations express somatically mutated \( \lambda_1 \) L chains. Representative \( \lambda_1 \) L chain sequences and predicted amino acid sequences from days 5 to 7 (A) or 9 to 14 (B) of the primary response or days 3–7 of the secondary response (C). Sequences are listed in chronological order from the indicated day of the response and are grouped according to phenotype as follows: CD138\(^+\), B220\(^-\)/CD138\(^+\); B220\(^-\), B220\(^+\)/CD138\(^-\); B220\(^+\), B220\(^+\)/CD138\(^+\); CD138\(^+\) memory responders, B220\(^-\)/CD138\(^+\) cells from the secondary response. Single (PI, CD4, CD8, F4/80, IgD, NP\(^+\)) cells of each subset were sorted from animals at various days of the response into a cDNA reaction mixture, subjected to two rounds of PCR using nested \( \lambda_1 \) L chain-specific primers, and the PCR products were sequenced. Frequency of obtaining a PCR product for each population is as follows: CD138\(^+\), 63%; B220\(^-\), 50%; B220\(^-\), 10%; and CD138\(^+\) memory, 65%. Sequences shown are from individual cells and are representative of the entire data set. Nucleotides found to differ from the germline sequences are indicated by letters (dashes represent germline sequence) and the predicted amino acids are shown above. Summary of L chain mutations from days 5 to 7 (D; \( n = 87 \)) and days 9 to 14 (E; \( n = 75 \)) of the primary response. # cells, The total number of L chain sequences obtained from single cells with the proportion of sequences that were mutated and non-mutated shown in parenthesis. % total, The percentage of the total number of base pairs sequenced that were mutated (reliable sequence from positions 26 to 89 for all sequences). # per mut seq, The number of mutations per mutated sequence and % replace, the percentage of mutations in CDR1 and 2 that are replacement mutations.
FIGURE 3. Each of the three subsets of NP-specific B cells express somatically mutated H chains during the primary response. A–C, Representative H chain sequences, displayed as described for Fig. 2, A–C. Single NP+ cells of each subtype were sorted from animals during the primary response into a cDNA reaction mixture, subjected to two rounds of nested PCR using VH186.2 H chain-specific primers, and the PCR products were sequenced. Frequency of obtaining a PCR product for each population is as follows: CD138+, 42%; B220+, 17%; B220-, 6%; and CD138+ memory, 28%. Sequences shown are
expressed and not efficiently amplified at the single-cell level. This trend is exaggerated among the B220\(^2\) population, as it is composed of ~50% IgE\(^+\) B cells, which will not cross-react with the IgG-directed primers used in this study. The B220\(^2\) population may also express lower levels of Ig mRNA than their plasma cell or GC counterparts. Nevertheless, these mutational analyses clearly show that the B220\(^-\) subset initially emerges unmutated and develops somatic hypermutations with evidence for increased affinity for Ag, providing strong evidence for affinity-driven selection and GC experience in the development of the B220\(^-\) B cell compartment.

The B220\(^-\) B cell population dominates the Ag-specific population in the BM

We next analyzed the emergence and phenotype of the NP\(^+\) population in the BM, an established site of Ag-specific B cell migration (1, 28, 29, 33–35). By day 7, an NP\(^+\)IgD\(^-\) population emerges abruptly above background levels to near maximal numbers (Fig. 4, A and B; 0.4% total BM). There is a gradual increase in NP-specific B cells from day 7 until maximal numbers are reached at day 21 (0.9% total BM). Cell numbers then decrease to levels that are maintained for at least 8 wk (20%

The B220\(^-\) B cell population is the dominant NP-specific B cell population in the BM. Animals were immunized as detailed for Fig. 1; BM harvested by aspirating both femurs and cells were labeled for flow cytometry. A, The NP-binding B cell population in the BM. Initial exclusion of PI-positive cells, T cells, and macrophages and gating of NP\(^+\) IgD\(^-\) B cells was performed as in Fig. 1A. B, Cellular dynamics of the NP-binding B cell compartment. Total numbers of NP-binding IgD\(^-\) B cells were calculated using frequencies obtained via flow cytometry and total BM cell numbers obtained after sacrificing animals. Each time point is representative of at least three animals with the means ± SEM displayed. C and D, Identifying subsets of Ag-specific B cells. NP-specific B cells (PI\(^-\), CD4\(^-\), CD8\(^-\), F4/80\(^-\), IgD\(^-\), NP\(^+\)) were analyzed for the expression of CD138 (PE-281.2) and B220 (FITC-RA3-6B2) at the days of the response indicated. D, Cellular dynamics of the three NP-binding B cell subsets. Numbers of cells with the indicated phenotypes were calculated as in B at the indicated days of the response. Each time point represents the mean ± SEM of at least three animals.

Summary of H chain mutations from days 5 to 7 (D: \(n = 32\)) and days 9 to 14 (E; \(n = 77\)) of the primary response. \# cells, % total, \# per mut seq, and % replace as in the legend to Fig. 2 and % W33L, the percentage of mutated sequences containing the affinity-enhancing amino acid change of tryptophan to leucine at position 33 (reliable sequence from positions 19 to 94 for all sequences).
of maximal levels). Thus, the B220− population accounts for the vast majority of NP-specific B cells in the BM throughout both the primary response and the quiescent memory phase (>90%; Fig. 4, C and D) and develops with kinetics that are distinct from their expansion upon secondary challenge. The B220−/CD138− population accounts for only a small portion of the NP-specific B cells in the BM (<0.003% of total BM through day 28), which is similar to reported frequencies of Ab-secreting cells in the BM (28, 29).

These data are consistent with a migration of B220− NP-specific B cells to the BM following primary immunization, where they persist as a substantial cellular reservoir for Ag-specific B cell memory.

The B220− population develops as two cellular subsets

Expression of the integrin CD11b and surface IgE divides the B220− memory population into two cellular subsets, CD11b+IgE− and CD11b−IgE+ (2), but the balance of these subsets in the quiescent memory compartment and their development during the primary response is not known. The cellular composition of the B220− population is most clearly investigated within the BM, where the B220− population accounts for >90% of NP-binding B cells during the primary response (Fig. 4C). Although the background of the CD11b+IgE− subset is 10-fold greater than that of the CD11b+IgE+ subset, both B220− subsets clearly emerge by day 7 in the BM (Fig. 5A). These two subsets persist at substantial levels for at least 8 wk. Both subsets of the B220− population also emerge rapidly in the spleen, with a CD11b+IgE− population composed of the B220− and the CD138− populations present as well (Fig. 5B). Interestingly, the second increase in NP+ cells in the spleen from days 14 to 21 is attributed mainly to the CD11b+IgE− subset. Thus, the two cellular subsets of B220− memory B cells develop during the primary response, not as a consequence of secondary encounter with Ag, and make up similar fractions of the quiescent memory compartment.

The B220− subset has a non-GC phenotype

Phenotypic markers can be used to discern GC cells from Ab-secreting cells and non-GC cells in the spleen by flow cytometry. Both the CD11b++ and IgE+ subsets bind higher levels of PNA than resting B cells, which does not allow us to distinguish them from GC cells (Fig. 6A). The phenotype of the CD11b++ and IgE+ subsets in the spleen and BM are identical, and samples from the BM are displayed here due to the absence of the B220+ and CD138− subsets. GL7 has more recently been used to identify GC B cells (23) and is highly expressed on a major fraction of B220− GC cells and is not expressed on either the CD11b++ or IgE+ subsets (Fig. 6B). BLA-1 has also been shown recently to identify GC B cells (25) and although it is highly expressed on B220− cells, it is present at greatly reduced levels on either CD11b++ or IgE+ cells (Fig. 6C). CD24 (HSA) has been used previously to label GC B cells (24) and is also expressed on B220− cells but not on CD11b++ or IgE+ cells (Fig. 6D). GC B cells have been shown to lack CD43 (leukosialin or Ly-48) (25), which is expressed at high levels on both CD11b− and IgE− subsets (Fig. 6E). Thus, although mutational analyses indicate that the B220− subset has transited the GC, phenotypic analysis indicates that B220− B cells are not found in the GC, implying that the down-regulation of B220 and expression of CD11b or IgE are post-GC events.

The B220− subset is not found in GC in situ

In situ analysis allows us to assess GC localization without the tissue disaggregation required for flow cytometric analysis. Using three-color laser scanning confocal microscopy, we can detect Ag-specific GC as IgD− regions that bind NP (red) within IgD+NP− B cell follicles (cyan; Fig. 7A–C). B220 was observed to be expressed on an overwhelming majority of IgD+NP+ GC B cells (red, Fig. 7D; yellow Fig. 7E), suggesting that the NP B220− population is not found in the GC at day 14. This is consistent with both our phenotypic analyses and previous studies reporting that GC B cells are B220− (25). Serial sections through the same GC show that the NP CD11b− subset is not found in the NP+ GC, IgD− B cell follicle, or CD11b+ MZ (MZ, Fig. 7F). The splenic red pulp is the area outside of the CD11b+ MZ and B220− B cell follicles and can be seen readily at lower power (interspersed B220+IgD− regions in Fig. 7A). The red pulp contains very few B220− B cells and the majority of NP+CD138− cells at day 14 (36, 37) (example shown in Fig. 7G). The majority of NP B220− CD11b− cells at day 14 were also found to be scattered throughout the red pulp. Fig. 7H shows an example of the NP B220−CD11b− subset found in the red pulp (colocalizes as
The high-lighted area is shown in greater detail in Fig. 7 with yellow and not associated with the B220 follicle). The highlighted area is shown in greater detail in Fig. 7I with NP+ B220− CD11b+ cells indicated by yellow arrows. These data are consistent with our phenotypic analyses and together clearly demonstrate that the B220− subset is not found in the GC at day 14, a time at which this subset is mutated with evidence for selection. Thus, the B220− subset appears to be a product of the GC, which undergoes a phenotypic alteration (down-regulation of B220 and expression of CD11b or IgE) upon exit from the GC.

The phenotype of the CD11b++ and IgE+ B cell subsets imply unique responses to Ag

Ag-driven B cell expansion depends not only on signals transmitted through the B cell receptor (BCR), but also on the contribution of regulatory molecules at the cell surface. An isoform of the tyrosine phosphatase CD45 is expressed at high levels on B220+ B cells, at intermediate levels on the CD11b++ and CD138+ populations, and at low to negative levels on the IgE+ subset (as detected by the Ab 30F.11, capable of recognizing all forms of CD45; Fig. 8A). CD79b/Igβ, the signal transduction component of the BCR, is expressed at high levels on the B220+ and CD11b++ populations and at low to negative levels on both the CD138+ and IgE+ populations (Fig. 8B). CD19, the signal transduction component of complement receptor (CR) 2, is expressed at high levels on B220+ B cells, down-regulated on the CD138+ population, and absent on both the CD11b++ and IgE+ subsets (Fig. 8C). The expression of CD21, the complement-binding component of CR2, differs from that of CD19. CD21 was expressed at high levels on the CD11b++ subset and down-regulated on the CD138+ and IgE+ populations (Fig. 8D). CD22, a negative regulator of BCR-mediated signaling, was expressed at high levels on the B220+ population and down-regulated on the CD138+ and B220+ populations (Fig. 8E). Together, these results predict that the B220− and both B220− memory B cell populations may differ in their activation requirements and the ability to respond to Ag and highlights a functional division of B cell memory.

**Discussion**

We have recently shown that a novel B220− memory population expands upon secondary challenge to dominate the memory response (2). How this subset develops is not clear and cannot be inferred from our previous work. Here, we show that this novel
FIGURE 7. In situ localization reveals that the B220− subset is not found in the GC. Spleens from mice sacrificed 14 days postprimary injection with NP-KLH were frozen and cryosections were prepared and stained for imaging via laser scanning confocal microscopy. A, NP binding and the splenic microenvironment. The ×10 image was stained with TR-IgD (cyan), FITC-B220 (green), and APC-NP (red). GCs can be seen as IgD− B220+ (green) and NP-specific GCs as IgD− B220+ NP+ (colocalize as yellow/orange). B, Schematic of GC highlighted in Fig. 6A. Indicated are the red pulp (RP), MZ, B cell follicle (B foll), and GC. C, IgD expression and Ag binding delineates the GC. Image of highlighted NP-specific GC in Fig. 6A stained with TR-IgD (cyan), APC-NP (red), and FITC-B220 (green; shown in Fig. 6D). B cell follicle is seen as IgD− NP− (cyan) and GC as IgD− NP+ (red). D and E, GC B cells are B220−. In the GC shown in Fig. 6C, IgD− B220− GC cells are seen as red (D) and NP− B220− GC cells are seen as yellow (E). F, The CD11b−NP− subset is not found in the GC. Serial section through the GC highlighted in Fig. 6A with APC-NP (red) and CD11b (green) staining displayed. The MZ is highlighted by CD11b expression. G, Ab-secreting cells in the red pulp. Image of red pulp area of the spleen with rhodamine-CD138 (red),
B220− subset, and its composite CD11b++ and IgE+ subsets, develops and persists following initial encounter with Ag in both the spleen and BM. Mutational analysis indicates that the B220− population has a GC phase in its development, whereas phenotypic and in situ analysis indicates that the B220− subset is not found in the GC, implying that the loss of B220 is a post-GC event. This recirculating subset localizes to the red pulp of the spleen, not the MZ and dominates the Ag-specific B cell compartment in the BM. Phenotypic analyses distinguish the B220− population from other atypical B cell subsets and indicates that these memory B cells may display unique responses to Ag recall. This current study quantifies the emergence of a novel post-GC B220− B cell population following primary immunization, demonstrating the development and maintenance of B cell memory in multiple, distinct cellular compartments.

**Affinity maturation of the B220− population**

Somatic hypermutation of immunoglobulin genes within the B220− population provides the clearest evidence of their GC experience and status as memory cells. Somatic hypermutation in the absence of histological GC has been reported (5, 6); however, the greater weight of evidence indicates that mutation proceeds in the GC microenvironment (8, 17, 38) and the loss of GC in numerous animal models decreases mutational activity (9–14). Thus, the somatic hypermutation and evidence for selection observed among the B220− subset as early as 9 days postprimary immunization most likely indicate a GC phase in their development and not occurring outside of the GC environment. The affinity-enhancing position 33 change is an indication of Ag-driven selection (32) and is seen among the majority of B220− B cells (68%), indicating that recruitment into the B220− compartment is affinity based. The B220− B cells have a lower penetrance of this affinity-increasing change at days 9–14 in vivo, suggesting that many of these cells are still undergoing selection within the GC environment. The phenotype of the B220− subset also indicates that they are not found in the GC, implying that the loss of B220 is a post-GC event. Thus, the B220− population has a GC phase in its development during the primary response, and the decision to

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**FIGURE 8.** The B220− B cell subsets lack B cell signaling molecules. Small resting B cells, CD138+, B220+, CD11b−, and IgE+ populations were gated as described in the legend to Fig. 7. The level of each molecule of interest on [CD4, CD8, F4/80]− (primarily T cells) is shown in white as negative controls; the negative level of the isotype-matched anti-CD21 Ab is shown as the negative for staining with the anti-CD45 Ab. The expression of CD45 (A), CD79b (B), CD19 (C), CD21 (D), and CD22 (E) is shown for each population with mean fluorescence intensity shown on each plot.
exit the GC and down-regulate B220 may be based on affinity for Ag.

**Localization of memory B cells**

The B22° population is a major component of the splenic post-GC B cell memory compartment. In situ, the CD11b⁺⁺ subset is not found in the GC at day 14, but is seen primarily in the red pulp. One surprising result from these localization studies is the lack of Ag-specific B cells in the MZ of the spleen at day 14, as MacLennan and colleagues (37) have shown that the MZ in rats is a major site of memory B cell localization. The large MZ population they describe is seen when animals are primed with carrier and reimmunized with hapten carrier, thus rendering T cell help nonlimiting. However, when they use a single priming protocol, as in our study, the MZ population is dramatically reduced when compared with carrier-primed animals and therefore may have escaped our detection. The red pulp localization of the splenic B22° B cells is more consistent with a recirculating memory B cell compartment primed for Ag surveillance and a rapid recall response.

The BM is another site of Ag-specific B cell localization (1, 28, 29, 33–35). Previous studies have focused primarily on the long-lived Ab-secreting population. This population is also observed in the current study, accounting for <0.005% of the total BM through day 28, which is consistent with previous studies using the NP system (28, 29). The B22° population, however, represents the vast majority of memory cells in the BM, accounting for nearly 1% of total BM cells at day 21. Although the rapid emergence and peak of the B22° population is consistent with migration from the spleen, it is not clear why this population would preferentially migrate to the BM. One possibility is that this migration is guided by the up-regulation of CD11b and expression of the β2 integrin unique to this population. It is also possible that this specialized microenvironment contains the requisite growth factors for long-term survival of the B22° B cell compartment. Nevertheless, the BM appears to provide a significant reservoir of these B22° memory response precursors.

**Phenotype of memory B cells**

The CD11b⁺⁺ subset is a novel B cell population distinct from previously reported atypical B cell populations. The CD11b⁺⁺ subset shares some phenotypic characteristics with the B-1 B cell population, but they differ in that splenic B-1 cells are CD5⁺, B22°⁻/⁻, HSA⁻⁻, IgM⁺, IgD⁻/⁻, and CD11b⁻, whereas the CD11b⁺⁺ subset is CD5⁺, B22°⁻, HSA⁻, IgM⁺, IgD⁻, and CD11b⁺⁺ (Refs. 39–42 and data not shown). In addition, the CD11b⁺⁺ subset dominates the Ag-specific population in the BM, while few B-1 cells are found in adult BM (40). Moreover, it has been shown that B-1 B cells do not respond to the T-dependent hapten NP used in this study (43). A population of biphenotypic cells with characteristics of both macrophages and B cells has also been recently described (44, 45). Although this population expresses CD11b, they are B22°⁻, IgM⁺, IgD⁻, CD5⁻, and F4/80⁻, whereas the CD11b⁺⁺ population does not express any of these markers. A population of B22°⁻ CD19⁺ B cells exists in the quasimonoclonal mouse model (46–48). This population, however, does not express CD11b and appears much larger by forward light scatter (48) than the CD11b⁺⁺ population described here. The lack of CD24 on B22° B cells is reminiscent of Klinman’s memory response precursors that preexist Ag challenge (49, 50). Although we have no evidence for their preexistence before Ag challenge, we propose that these unique memory B cells are a product of the primary response GC reaction that has separate and distinct activation requirement for their response to Ag recall.

The possibility of the IgE⁺ subset being non-B cells such as mast cells or eosinophils with NP-specific Ig bound to the surface is unlikely based on a number of observations. Mast cells and eosinophils circulate in a resting or immature state until they are recruited to sites of local tissue reactions or allergic inflammation (primarily mucosal and connective tissues for mast cells and airways for eosinophils) where they mature to an effector state and have a life span of only days (51–54). In contrast, the IgE⁺ B cell subset is found in large numbers in the BM and persists at levels 10–100-fold over background in the spleen and BM for at least 8 wk in the absence of further antigenic challenge. In addition, electron microscopy studies of sorted NP⁺ B22° B cells did not reveal any cells with eosinophil or mast cell morphology (data not shown). Phenotypically, the IgE⁺ subset does not express the Fce receptor CD23 (data not shown). Finally, although the IgE⁺ subset lacks a number of typical B cell markers, most notably CD79b, CD19, CD21, and B220, they are phenotypically most similar to the CD138⁺ Ab-secreting B cell population in these same respects.

**Response to Ag recall**

Cell surface phenotype can offer insight into the activation requirements of B cell memory subsets, which are thought to differ from their naive counterparts in their activation requirements and proliferative capacity (55–57). The CD11b⁺⁺ subset expresses surface IgG, expresses high levels of CD79b, and proliferates upon transfer with Ag into RAG1⁻/⁻ mice (2), implying that this memory population can respond to Ag via BCR-mediated interactions. However, the role of coreceptor-Ag interactions appears to differ among CD11b⁺⁺ and B22° memory cells. Although the CD11b⁺⁺ subset expresses the complement-binding component of CR2, CD21, it lacks the signal transduction component, CD19, responsible for augmenting the BCR-mediated signal. The expression of complement CR3 (CD11b/CD18), however, may indicate that this subset requires a different set of Ag-complement interactions. The CD11b⁺⁺ subset does not express CD22, which acts as a negative regulator via recruitment of SHP1 (58–62). However, this lack of CD22 may not have a significant effect on the activation of the CD11b⁺⁺ subset in the absence of CD19, as a recent study has shown that CD22 exerts its negative effects by acting primarily upon CD19 (63). Thus, the surface phenotypes of the individual B cell memory subsets may predict unique and specialized activation requirements for each subset.

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