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*J Immunol* 2006; 176:4706-4715; doi: 10.4049/jimmunol.176.8.4706

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Memory B cells help to protect the host from invading pathogens by maintaining persistent levels of Ag-specific serum Ab and generating rapid Ab responses upon re-exposure to Ag. Unambiguous identification of memory B cells has been a major obstacle to furthering our knowledge concerning both the development of B cell memory and secondary Ab responses due to an absence of specific cell surface markers. Germinal centers (GCs) are thought to be the major site of Ig hypermutation and Ag-driven selection of memory B cells. To develop a model that would identify GC-derived memory B cells, we generated transgenic mice that expressed cre recombinase in a GC-specific fashion. Interbreeding these mice with the cre-reporter strain, ROSA26R, produced progeny in which β-galactosidase (β-gal) was permanently expressed in B cells of the GC-memory pathway. Analysis following immunization with (4-hydroxy-3-nitrophenyl)acetyl coupled to chicken γ globulin showed that long-lived β-gal⁺ B cells exclusively contained somatically mutated λ₅ V regions and were capable of producing Ag-specific Ab-forming cell (AFC) responses that were >100-fold higher than those afforded by β-gal⁻ B cells following adoptive transfer to naive hosts. Secondary challenge of immune mice showed that only ~20% of secondary AFCs expressed β-gal. Interestingly, we found that somatic hypermutation of rearranged λ₅ V regions within secondary AFCs showed a strong correlation with β-gal expression, suggesting that nonmutated B cells contribute significantly to secondary Ab responses. This model should provide useful insights into memory B cell development, maintenance, and differentiation following immunization or pathogenic infection. The Journal of Immunology, 2006, 176: 4706–4715.

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of 10 matings. GC-cre (GCC) transgenic founders (lines GCC1.6, GCC816, and GCC158) were derived in the C57BL/6 background at Rockefeller University (New York, NY) (GCC816 and GCC1.6) or Emory University Transgenic Core Facility (Atlanta, GA) (GCC158). ROSA26R, and GCC mice were genotyped via PCR of genomic DNA derived from tail-tip biopsies. Primers for ROSA26R PCR have been described (22). Cre-specific primers are as follows: cre-forward, 5′-ACACCCGTTTGAATG AGCAGCGCCTGG-3′; and cre-reverse, 5′-TATCCGCCATCACTACGAGCAGCAG-3′. All GCC mice were maintained in accordance with C57BL/6 and housed under specific pathogen-free conditions at the Emory Vaccine Center. The succinimide ester of the hapten (4-hydroxy-3-nitrophenyl)acetyl (Cambridge Research Biochemicals) was coupled to chicken γ globulin (Sigma-Aldrich), and the NP-CG molar ratios were determined via spectrophotometry. We used NP2-CG and NP6-CG for these studies. Primary immune responses were induced by injecting 50 μg of alumin precipitated NPCG. Secondary responses were induced via tail-vein injection of 20 μg of soluble NPCG in PBS. All studies were approved by Emory University’s Institutional Animal Care and Use Committee.

β-Gal detection, Abs, and flow cytometry

Spleens were harvested from immunized and naive animals, cleared of RBCs (RBC lysis buffer; Sigma-Aldrich), and washed twice with staining buffer (2% FBS; 0.01% Na azide in PBS). For detection of β-gal activity, 1–10 × 10⁶ RBC-cleared splenocytes were washed once with 1× PBS, resuspended in 0.2–0.5 ml of PBS, and warmed to 37°C. Next, an equal volume of 250 μM fluorescein-di-β-galactopyranoside (FDG) (Molecular Probes) in distilled H₂O (prewarmed to 37°C) was added to the cells, mixed, and incubated at 37°C for 2 min in an air incubator. The loading was quenched by the addition of 10 volumes of cold PBS. For Ab staining, 1–2 × 10⁶ splenocytes were next incubated on ice for 20 min with optimal concentrations of anti-B220-allophycocyanin (Caltag Laboratories), CD8-PE (JC11), CD95-PE, GL-7-biotin, CD138-PE (clone 281.2), CD4-PE (BD Biosciences), CD8-PerCP, CD8-PE, CXCR5-PE, CD19-PE, CD11c-PE, F4/80-PE, GR1-PE (all from BD Biosciences), or peanut agglutinin (PNA)-biotin (Sigma-Aldrich). Biotinylated reagents were detected with either 5 μg/ml streptavidin-allophycocyanin (Molecular Probes) or streptavidin-PerCP (Molecular Probes). All cells were finally labeled with anti-β-gal PE (Invitrogen–gal) or anti-β-gal PE (Invitrogen–gal at variable frequencies (10–90%) in both T and B cell lineages and evaluated by FACS and FACSCalibur flow cytometers (BD Biosciences) runnning FlowJo software (Tree Star).

ELISPOT assay

ELISPOT assays were conducted as described (23), with the exception that 96-well nitrocellulose plates (Millipore) were coated overnight with 20 μg/ml NPCG in 50 μl of PBS. Spots were visualized using an ELISPOT reader (Cellular Technologies) and counted manually.

Cell sorting and adoptive transfers

For cell-sorting experiments, RBC-cleared splenocytes were loaded with FDG and stained with Abs as described above. For isolation of memory B cells, B220−/− Synd-1−/− cells from immune (days 60–120 postimmunization (p.i.)) GCCxR26R mice were sorted into β-gal− and β-gal+ fractions. CD4−T cells from immune (>35 days p.i.) cre-negative GCCxR26R mice were negatively sorted by excluding all cells that expressed CD19, CD8, Synd-1, CD11c, F4/80, and GR1. Two- and three-way cell sorting was performed on a FACSAria (BD Biosciences) or MoFlo (DakoCytometry) cell sorter, respectively, each with high-speed capabilities (Emory University Flow Cytometry Core). Sorted cell purities ranged from 85 to 98%. For adoptive transfers, sorted splenocytes were combined, pelleted, and resuspended in 200 μl of PBS. Cells suspensions were administered to the tail vein of Rag2−−/− recipients. At 48 h following transfer, recipients were challenged i.p. with 20 μg of NPCG in PBS.

PCR and DNA sequencing

Genomic DNA was isolated using standard proteinase K digestion followed by salt extraction and precipitation with an equal volume of isopropanol. Seminested PCR (High-Fidelity FastStart PCR kit; Roche) was used to amplify the λ L chain V-J region using primer sequences previously reported (24). PCR products were purified (PCR Clean kit; Qiagen) and digested overnight with BamHI and HindIII (NEB). Digested products were agarose purified (Qiagen), subcloned into pBluescript KS−− (Invitrogen Life Technologies), and used for transforming TOP10F bacteria (Invitrogen). λ phage plaques for nucleic acid templates for each single primer were generated on Petri dishes by using one copy of PCR using M13-F and M13-R primers followed by purification of PCR products (Millipore). Sequencing reactions were performed in 10-μl volumes with one-fourth the manufacturer’s recommended enzyme concentra-
not responsible for the variable expression observed from the truncated I-E<sub>n</sub><sup>a</sup> promoter. Considering the relatively low penetrance (~20%) of aberrant β-gal expression, we chose to prescreen the peripheral blood of naive GCCxR26R offspring and excluded individuals that exhibited β-gal expression before experimentation.

**Peyer’s patches (PPs) contain large, constitutive GCs due to the continual recruitment of GC precursor cells by persistent gut Ags (27).** Therefore, our model predicts that PPs from naive GCCxR26R mice would contain significant frequencies of β-gal<sup>+</sup> B cells, whereas spleens from these animals would not. Analysis of PPs from naive GCCxR26R mice confirmed a high frequency of B cells that bound PNA (15.7–16.7%; n = 2), indicating ongoing GC reactions (Fig. 2a). Accordingly, β-gal expression also was found in a high frequency of PP B cells (7.8–8.2%). In contrast, both PNA binding (0.29–0.35%) and β-gal expression (0.25–0.44%) among splenic B cells was minimal. These results suggested that the development of β-gal<sup>+</sup> B cells was associated with concurrent GC reactions.

To investigate the extent and kinetics of β-gal expression following immunization, we immunized cohorts of GCCxR26R mice and single transgenic R26R controls with 50 μg of alum-precipitated NPCG and analyzed splenic B cells for β-gal expression by flow cytometry at various time points following immunization. We found that β-gal<sup>+</sup>B220<sup>+</sup> cells were evident in the spleen 8 days following immunization of GCCxR26R mice and increased in number until day 12, accounting for 2.1% ± 0.38 of total splenocytes (3.5% ± 0.54 of B220<sup>+</sup> cells) at their peak (Fig. 2b). This was followed by a modest contraction phase that reached steady-state levels by day 26 p.i. Immunized single transgenic littermate controls failed to express β-gal at all time points examined (data not shown). The kinetics of β-gal expression was similar to PNA<sup>+</sup> GC B cell development; however, the frequency and total number of β-gal<sup>+</sup> B cells per spleen was consistently less than PNA<sup>+</sup> B cells during the early to intermediate stages of the primary response. However, during the immune phase of the response (>4 wk p.i.), the number of β-gal<sup>+</sup> B cells was higher than PNA<sup>+</sup> B cells, suggesting the development of β-gal<sup>+</sup> memory B cells over time (data not shown).

**β-Gal expression does not mark all GC B cells**

To determine the anatomical location of β-gal expression, we incubated 8-μm spleen sections taken from GCCxR26R and single transgenic control mice immunized 16 days previously with NPCG overnight in 5-bromo-4-chloro-3-indolyl β-D-galactoside solution to reveal β-gal expression. As shown in Fig. 3a, β-gal-stained GC structures can be seen throughout the tissue section. Additionally, diffuse staining scattered throughout the section can be seen, indicating that not all β-gal<sup>+</sup> cells are found within GC. Some degree of extramural staining was expected, because expression from the truncated I-E<sub>n</sub><sup>a</sup> promoter was reported in the red pulp and interdigitating DCs (25).

Next, we determined the extent of correlation between β-gal expression and markers associated with GC B cells following immunization with NPCG. In addition to PNA-binding, GC B cells are typically characterized by increased expression of GL7 and CD95 (Fas), and decreased expression of CD38 (28–30). Flow cytometry analysis of β-gal<sup>+</sup> B cells from the spleens of GCCxR26R mice immunized 10 days previously showed that 22.74% ± 1.35 bound PNA, 13.14% ± 5.26 were CD38<sup>bright</sup>, and expression of GL7 and CD95 was detected in 16.0% ± 3.76 and 20.33% ± 4.7 of β-gal<sup>+</sup> B cells, respectively (Fig. 3b). In addition, β-gal<sup>+</sup>B220<sup>+</sup> cells were uniformly positive for CD19 and CXCR5 expression and did not express CD4 or CD8 (data not shown). The discordance between β-gal expression and typical GC markers is in agreement with the data in Fig. 3a, which demonstrated diffuse β-gal expression outside of GC regions. Additionally, this finding was not unique to the time point analyzed. Analysis on days 8, 16, 26, and 35 p.i. showed that, whereas the frequency of both PNA<sup>+</sup> and β-gal<sup>+</sup> B cells waxed and waned, the frequency of PNA<sup>+</sup> B cells within the β-gal<sup>+</sup> B cell fraction did not substantially change over the course of the primary response (data not shown). These results demonstrate that β-gal does not mark all PNA<sup>+</sup> GC B cells.

Given that β-gal expression by splenic B cells was not seen in naive mice and was observed only following immunization (Fig. 2), we sought to exclude the possibility that β-gal<sup>+</sup>PNA<sup>+</sup> B cells were primary AFCs. We analyzed splenocytes taken from GCCxR26R mice 8 days p.i. for expression of B220, β-gal, and syndecan-1 (CD138; Synd-1), a marker of plasma cell differentiation. As shown in Fig. 3c, B220<sup>+</sup>Synd-1<sup>+</sup> cells accounted for ~0.5% of splenocytes 8 days p.i. Among these, 9–24% showed β-gal expression. Conversely, B220<sup>+</sup>β-gal<sup>+</sup>Synd-1<sup>+</sup> cells represented only 3–9% of the total β-gal<sup>+</sup> population at this time. The presence of small numbers of β-gal<sup>+</sup> AFCs most likely represents early plasma cell emigrants from concurrent GC reactions, as recently reported by Blink et al. (31). However, these data demonstrate that the majority of β-gal<sup>+</sup>PNA<sup>+</sup> cells were Synd-1<sup>+</sup> and...
hence did not participate in the primary AFC response following immunization.

**β-gal**\(^+\) PNA\(^+\) GC B cells contain mutated λ\(_1\) V regions

The phenotype of β-gal\(^+\) B cells was unexpected and suggested that many did not participate in GC reactions. Alternatively, the lack of GC marker expression by β-gal\(^+\) B cells may have reflected a latter stage of GC development, one in which expression of GC markers down-regulated before emigration from GC. To better understand the phenotype of the β-gal\(^+\) B cell populations, we determined which populations of β-gal\(^+\) B cells underwent hypermutation of their rearranged λ\(_1\) V region loci following immunization. The hypermutation of IgV regions affords GC B cells with a competitive advantage during Ag-driven selection by increasing BCR affinity for Ag. The frequency of hypermutation within hypervariable regions (which encode for complementarity determining regions (CDRs)), vs those in framework regions, are reliable indicators of a cell’s fitness for Ag-driven selection, because CDRs possess the majority of residues responsible for Ag-binding (32). Additionally, affinity-enhancing mutations must be nonsilent (i.e., cause amino acid replacements). Therefore, these two parameters are often predictive of Ag-driven selection. We immunized cohorts of GCCxR26R mice with NPCG, harvested and pooled their spleens, and sorted B220\(^+\) B cells with β-gal\(^+\) PNA\(^+\), β-gal\(^+\) PNA\(^-\), and β-gal\(^+\) PNA\(^-\) phenotypes 8 or 15 days following immunization (n = 3 per time point). Genomic DNA was harvested from each population, and rearranged λ\(_1\) V gene segments were amplified by seminested PCR. Due to the predominant use of λ\(_1\) L chains in mice of the Igh\(^b\) allotype during the primary response to NP, this strategy allowed us to selectively amplify V regions of NP-specific B cells (33, 34). The products were cloned and individual colonies were then chosen for sequence analysis. To reduce the likelihood of over-amplification of individual clones, the number of PCR cycles was titrated to the minimum number that allowed sufficient product generation for subsequent cloning steps. Genomic DNA from the spleen of a naïve GCCxR26R mouse was isolated simultaneously and treated together with the sorted populations to control for PCR error rates.

The mutation data are summarized in Table I, showing that 60% of sequences in GC or that they did not survive long enough within GC to accumulate significant mutations. In contrast, 58% of sequences...
recovered from β-gal\(^{-}\) PNA\(^{+}\) B cells at day 8 p.i. were mutated, and this frequency rose to 67% by day 15 p.i. A high frequency (73–82%) of mutations in β-gal\(^{-}\) PNA\(^{+}\) B cells gave rise to amino acid replacements, and the percentage of mutations that fell within CDR rose from 47% on day 8 to 73% by day 15. However, the mutation frequency of β-gal\(^{-}\) PNA\(^{+}\) B cells remained stable from days 8–15 p.i. (0.443 and 0.39 on days 8 and 15, respectively). The frequency of mutated β-gal\(^{-}\) PNA\(^{+}\) B cells rose from 45% on day 8 to a frequency similar to PNA single positive B cells by day 15 (75%). However, the number of mutations in β-gal\(^{-}\) PNA\(^{+}\) B cells that fell within CDR ranged from 100% at day 8 p.i. to 80% 15 days following immunization and gave rise to amino acid replacements 80–90% of the time. In contrast with β-gal\(^{-}\) PNA\(^{+}\) B cells, whose mutation frequency did not change significantly, the mutation frequency of β-gal\(^{-}\) PNA\(^{+}\) B cells increased 1.8-fold from day 8 to day 15, although this increase was not statistically significant. The presence of somatic hypermutation within rearranged \(\lambda_1\) V regions demonstrates that β-gal\(^{-}\) B cells with a GC phenotype (PNA\(^{+}\)) participated in GC reactions. Furthermore, the pattern and frequency of mutations from both β-gal\(^{-}\) PNA\(^{+}\) and β-gal\(^{-}\) PNA\(^{+}\) B cells suggested that these cells were under Ag selection pressures.

\(\beta\)-gal\(^{+}\) memory B cells contain mutated \(\lambda_1\) V regions

Fig. 2b clearly shows that β-gal\(^{+}\) B cells persisted for extended periods of time following immunization. To determine whether the β-gal\(^{+}\) B cell population in immune GCCxR26R mice represented authentic memory B cells, we analyzed rearranged \(\lambda_1\) V gene segments from β-gal\(^{+}\) and β-gal\(^{-}\) B cells taken from immune GCCxR26R mice for evidence of hypermutation and Ag-driven selection. GCCxR26R mice were immunized with NPCG and sacrificed 60 days later or, alternatively, boosted with soluble Ag and rested for 50 days before sacrifice. B220\(^{+}\) cells were then sorted according to β-gal expression and sequenced as above.

The number of mutations per clone is shown in Fig. 4a, and population mutation frequencies are graphed in Fig. 4b. Seventy-nine percent (38 of 48) of clones from B220 \(\beta\)-gal\(^{+}\) B cells displayed up to five mutations per sequence. In contrast, B220 \(\beta\)-gal\(^{-}\) B cells displayed very few mutations; 80% (33 of 41) were unmutated, while the remaining 20% contained a single mutation each. This frequency did not significantly differ from that observed in rearranged \(\lambda_1\) loci from naive B cells (0.069 vs 0.052; \(p = 0.65\)). The mutation frequency in \(\beta\)-gal\(^{-}\) memory B cells was 8.1-fold higher than in β-gal\(^{-}\) B cells (0.560 vs 0.069; \(p < 0.001\)), and 10.7-fold higher than in splenocytes taken from a naive GCCxR26R control (0.560 vs 0.052; \(p < 0.001\)). Seventy-two percent of unique mutations from β-gal\(^{-}\) B cells yielded amino acid changes, and 58% of these fell within CDRs, a pattern indicative of Ag-driven selection. These results show that \(\lambda_1\) V region mutations are found almost exclusively within the β-gal\(^{-}\) B cell pool in immune GCCxR26R mice.

Memory recall responses are transferred by β-gal\(^{-}\) B cells from immune mice

Next, we determined whether β-gal\(^{-}\) memory B cells were capable of transferring memory responses to naive mice. We performed adoptive transfers in which \(5 \times 10^4\) purified β-gal\(^{-}\) Synd-1\(^{-}\) or

### Table I. Summary of GC B cell \(\lambda_1\) hypermutation

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Day after Immunization</th>
<th>No. Sequenced (% mut)</th>
<th>Mutation Frequency</th>
<th>No. per Mutated Sequence</th>
<th>% Replace</th>
<th>% CDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)gal(^{-}) PNA(^{+})</td>
<td>d8</td>
<td>12 (58)</td>
<td>0.443</td>
<td>2.04</td>
<td>73</td>
<td>47</td>
</tr>
<tr>
<td>(\beta)gal(^{-}) PNA(^{+})</td>
<td>d8</td>
<td>12 (45)</td>
<td>0.322</td>
<td>2.0</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>(\beta)gal(^{+}) PNA(^{+})</td>
<td>d8</td>
<td>10 (40)</td>
<td>0.248</td>
<td>1.75</td>
<td>42</td>
<td>71</td>
</tr>
<tr>
<td>(\beta)gal(^{+}) PNA(^{+})</td>
<td>d15</td>
<td>10 (67)</td>
<td>0.390</td>
<td>1.57</td>
<td>82</td>
<td>73</td>
</tr>
<tr>
<td>(\beta)gal(^{+}) PNA(^{+})</td>
<td>d15</td>
<td>12 (75)</td>
<td>0.591</td>
<td>2.5</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>(\beta)gal(^{+}) PNA(^{+})</td>
<td>d15</td>
<td>11 (18)</td>
<td>0.193</td>
<td>3.0</td>
<td>50</td>
<td>67</td>
</tr>
</tbody>
</table>

\(a\) Total number of clones sequenced, with the percentage of sequences having mutations shown in parentheses.

\(b\) Frequency of unique nucleotide changes within the total number of base pairs sequenced.

\(c\) Average number of mutations within mutated sequences.

\(d\) Percentage of unique nucleotide changes that gave rise to amino acid replacements.

\(e\) Percentage of unique mutations that fell within CDRs 1, 2, and 3.
β-gal− Synd-1− B cells from immune GCCxR26R mice (days 60–120 p.i.) were transferred to immunodeficient RAG2−/− hosts (schematic in Fig. 5a). To provide a source of T cell help, each mouse received ~1 × 10⁶ negatively sorted CD4⁺ T cells from NPGC-immune R26R mice (>day 60 p.i.). In addition, each recipient also received 3 × 10⁵ splenocytes from naïve Ly5.1 mice to aid in lymphoid follicle formation in the RAG2−/− hosts. To control for possible contamination of small numbers of memory B cells within the CD4⁺ T cell fraction, one cohort of recipients received only CD4⁺ T cells from immune mice plus the naïve splenocyte fraction. Additionally, two groups of mice received nonfractionated splenocytes (5 × 10⁶) from immune GCCxR26R mice in which one group was given Ag and the other was left unchallenged. The recipients were challenged i.p. with 20 μg of soluble NPCG 48 h following cell transfer. We harvested spleens from each recipient 5 days following Ag challenge and analyzed them for the presence of IgG-secreting Ag-specific AFCs by ELISPOT analysis (Fig. 5b).

As expected, Ag-challenged recipients that received nonfractionated splenocytes from immune GCCxR26R mice contained elevated numbers of splenic AFCs capable of secreting NPCG-specific IgG (76 ± 10 spots per 10⁶ cells). We were unable to detect Ag-specific AFCs from the spleens of either unimmunized recipients of nonfractionated splenocytes, nor recipients of CD4⁺ T cells alone (<4 spots/10⁶ cells). In contrast, animals that received β-gal⁺ memory B cells contained high numbers of AFCs that secreted Ag-specific IgG (382 ± 45 per 10⁶). AFC detection in β-gal− recipient spleens was virtually absent (5.8 ± 4.9 per 10⁶). The lack of an AFC response from mice that received only CD4⁺ T cell help demonstrated that the responses detected in β-gal⁺ recipients was not a result of contaminating AFCs in the sorted T cell fraction.

Several recent reports demonstrating the development of memory B cells in the absence of ongoing GC prompted us to determine whether memory B cells were present at low frequencies in the β-gal− B cell fraction from immune GCCxR26R mice (35–37). To address this, we performed adoptive transfers as above, except 40-fold higher numbers (2 × 10⁶) of β-gal− memory B cells were transferred. This scheme allowed us to detect a low AFC response (132 ± 48 spots per 10⁶), demonstrating that Ag-specific B cells were indeed present in the β-gal− B cell fraction. Normalizing for the number of cells transferred, these results demonstrate that Ag-specific memory B cells were enriched by two orders of magnitude (116-fold) in the β-gal⁺ B cell fraction, compared with B cells that lacked β-gal expression (Fig. 5b).

**β-gal⁺ memory B cells undergo AFC differentiation upon secondary Ag challenge**

To determine whether β-gal⁺ memory B cells responded to secondary Ag challenge in situ, we challenged cohorts of NPCG-immune (>35 days p.i.) GCCxR26R and single transgenic littermate control mice with soluble NPCG and analyzed their spleens.
4 days later for β-gal and Synd-1 expression. Flow cytometry analysis showed that β-gal expression segregated into distinct B220\(^+\) and B220\(^{−}\) populations (Fig. 6a, top row). Further analysis showed the B220\(^{−}\)β-gal\(^+\) fraction expressed the AFC marker, Synd-1, while B220 \(^{−}\)β-gal\(^+\) cells did not. Interestingly, B220\(^{−}\)β-gal\(^+\) Synd-1\(^−\) cells did not account for the entire population of plasma cells that arose during the secondary response. As shown in Fig. 6a (middle row), β-gal was expressed in 20.4% ± 2.7 (n = 10) of the total Synd-1\(^−\) population. In addition to Synd-1 expression, both β-gal Synd-1\(^−\) and β-gal Synd-1\(^−\) cells showed increased forward scatter properties, compared with β-gal Synd-1\(^−\) and β-gal Synd-1\(^−\) cells, a property that is consistent with a plasma cell phenotype.

To determine which populations were capable of Ag-specific Ab production, we performed simultaneous three-way cell sorting to isolate each population of responding B cells (B220\(^{−}\)β-gal Synd-1\(^−\), B220\(^{−}\)β-gal Synd-1\(^−\), and B220 \(^{−}\)β-gal Synd-1\(^−\)) from the spleens of GCCxR26R mice 4 days following Ag recall and analyzed them directly ex vivo for NPCG-specific Ab production using plasma cell ELISPOT assays. Surprisingly, we found that B220\(^{−}\)β-gal Synd-1\(^−\) and B220\(^{−}\)β-gal Synd-1\(^−\) cells contained nearly identical frequencies of Ag-specific AFCs (4.8 \(\times\) 10\(^{4}\) vs 5.1 \(\times\) 10\(^{4}\) per 10\(^{5}\) sorted cells) (Fig. 6b). In contrast with the results obtained from Synd-1\(^−\) populations, B220 \(^{−}\)β-gal Synd-1\(^−\) B cells contained very few AFCs (~700 per 10\(^5\) cells), confirming Synd-1 expression as a faithful marker of plasma cell identity. B220 \(^{−}\)β-gal Synd-1\(^−\) cells were not plasmablasts that simply required additional time for differentiation to AFCs because, in contrast with Synd-1\(^−\) populations, we were unable to detect Ag-specific serum Ab following adoptive transfer to RAG2-deficient hosts (data not shown). Taken together, these data 1) confirm the Ag-specific nature of β-gal memory B cells, 2) demonstrate their ability to rapidly differentiate to AFCs in situ, and 3) revealed that 80% of secondary AFCs are not marked by β-gal expression.

Highly mutated λ\(_1\) V regions are restricted to β-gal\(^+\) AFCs following secondary Ag challenge

The data in Fig. 6 showed that ~80% of secondary AFCs were not marked by β-gal expression but were capable of Ag-specific IgG production. This suggested the possibility that only ~20% of memory B cells were marked by β-gal expression. If this were the case, we reasoned that β-gal\(^−\) and β-gal\(^+\) AFCs found following secondary challenge would display a similar distribution and frequency of somatic mutations within their \(\lambda_1\) V regions. To address this issue, we sorted B220\(^{−}\)β-gal Synd-1\(^−\), B220\(^{−}\)β-gal Synd-1\(^−\), as well as B220 \(^{−}\)β-gal Synd-1\(^−\) cells from GCCxR26R mice 4 days following secondary Ag challenge and sequenced the \(\lambda_1\) V region from each population as described above.

The number of mutations per clone is shown in Fig. 7a and mutations frequencies are shown in Fig. 7b. As expected, we found that β-gal\(^−\) secondary AFCs contained very high frequencies of mutated cells (91%). The average number of mutations per sequence was 4.14, and the overall mutation frequency was 1.47. A

**FIGURE 6.** Development of Ag-specific β-gal\(^+\) secondary AFCs following Ag recall. Cohorts of immune GCCxR26R and littermate control mice (≥35 days p.i.) were challenged i.v. with 20 μg of soluble NPCG. Spleens were harvested 4 days following Ag challenge and analyzed for the presence of β-gal\(^+\) AFCs by flow cytometry. a, The top row shows the presence of β-gal\(^+\) B220\(^+\) and β-gal B220\(^{−}\) populations following secondary Ag challenge. The histograms show Synd-1 expression (left) and forward scatter properties (right) of gated β-gal B220\(^+\) (solid line) and β-gal B220\(^{−}\) (dashed line) populations. The bottom row depicts Synd-1 expression among gated live splenocytes. Histograms depict β-gal expression (left) and forward scatter properties (right) among gated Synd-1 B220\(^{−}\) cells. Data shown are representative of three independent experiments (n = 3–4 mice per experiment). b, B220\(^{−}\)β-gal Synd-1\(^−\), B220\(^{−}\)β-gal Synd-1\(^−\), and B220 \(^{−}\)β-gal Synd-1\(^−\) cells indicated in the flow diagram were sorted 4 days following secondary challenge of immune GCCxR26R mice (day 60–120 p.i.) and analyzed for Ag-specific AFCs directly ex vivo by ELISPOT assay. Plotted are the number of IgG-secreting NPCG-specific AFCs per 10\(^5\) sorted cells. Each data point represents an individual animal. Data shown are the combined results from three independent experiments (n = 2–3 per experiment).

**FIGURE 7.** \(\lambda_1\) V-region hypermutation among secondary AFCs segregates with β-gal expression. a, Scatter plots show the number of mutations per clone found within \(\lambda_1\) V regions from B220 \(^{−}\)β-gal Synd-1\(^−\), B220\(^{−}\)β-gal Synd-1\(^−\), and B220 \(^{−}\)β-gal Synd-1\(^−\) cells. Each data point represents an individual clone. The bar graph in b depicts the mutation frequency of the indicated populations. The numbers in parentheses specify the number of unique of clones analyzed.
high frequency (77%) of these mutations led to amino acid replacements, and the majority (60%) of mutations were confined to CDR, demonstrating Ag-driven selection. These results provide additional evidence that β-gal + AFCs found after Ag recall are derived from authentic memory B cells. In contrast with β-gal + AFCs, the mutation frequency among β-gal − AFCs was significantly reduced (1.47 vs 0.36; p < 0.001). Although 61% of B220 + β-gal + Synd-1 + cells displayed mutations, the average number of mutations per sequence (1.09) was 3.8-fold less than that recovered from B220 + β-gal + Synd-1 + clones and never exceeded four mutations per sequence (compared with 68% of B220 + β-gal + Synd-1 + cells, which carried ≥4 mutations per sequence). Eighty-four percent of mutations within B220 + β-gal + Synd-1 + cells yielded amino acid replacements, and 52% fell within CDR. Taken together, these results indicate that, following secondary Ag challenge, mutated λ V regions are found predominantly within AFCs that expressed β-gal.

Analysis of mutation patterns from B220 + β-gal + Synd-1 − cells showed that these cells contain mutations that were quite different from B220 + β-gal + Synd-1 + AFCs. The majority (64%) of B220 + β-gal + Synd-1 − clones did not contain mutations. The distribution of mutations in β-gal + Synd-1 + B220 + cells was not indicative of Ag-driven selection. Only 20% of nucleotide mutations fell within CDR, although each mutation led to amino acid replacements. These results suggest separate developmental pathways for B220 + β-gal + Synd-1 − and B220 + β-gal + Synd-1 + cells (see Discussion).

Discussion
In a novel mouse model system, we induced permanent β-gal expression in GC B cells via cre-mediated recombination, which allowed us to detect both B cells participating in GC reactions and long-lived Ag-specific memory B cells that persisted for at least 4 mo following the initial antigenic stimulus. We demonstrate that β-gal + memory B cells contain three hallmark traits of B cell memory: 1) Ag-specificity, 2) hypermutated IgV gene segments, and 3) the ability to transfer memory responses following adoptive transfer to naïve hosts.

Previous studies by Shinall et al. (29) on the primary GC responses to SRBC demonstrated that frequencies of GC-specific markers and surface Ig isotype expression remained remarkably consistent during the course of the primary GC response. In agreement with these studies, we found that the frequencies of PNA +, GL7 +, CD95 +, and CD38low B cells that expressed β-gal remained constant throughout the primary response. However, we were surprised to find that only 20–30% of β-gal + B cells expressed GC-specific markers. Our analysis of rearranged λ V-region sequences from sorted β-gal + PNA + B cells showed that a small number of these cells contained a low frequency of somatic mutations (average of 0.22%), strongly suggesting that they did not represent late-stage GC B cells. The permanent nature of β-gal expression from the ROSA promoter indicates that β-gal + PNA + B cells either did not participate in GC reactions or did not survive within GC for sufficient time to undergo hypermutation or up-regulate receptors for PNA. Although the precise fate of these cells remains unclear at present, based upon mutation frequencies this population did not appear to participate in GC or develop into long-lived memory B cells. This is contrasted by β-gal − PNA − B cells whose mutation frequency at day 15 p.i. (0.591) was similar to that found within β-gal − B220 + memory B cells that persisted long-term after immunization (0.560).

The presence of mutated λ V gene segments is compelling evidence for the GC origins of memory B cells because 1) the process somatic hypermutation is largely restricted to GC reactions, and 2) inhibition of key events such as CD28 or CD40 signaling abolishes the formation of hypermutated memory B cells and greatly diminishes secondary Ab responses (11, 20, 35, 38–41). λ V region sequence analysis of β-gal + and β-gal − memory B cells from immune GCCxR26R mice showed that the presence of hypermutation correlated strongly with β-gal expression. Interestingly, two of three mice displayed a low clonal diversity among β-gal + memory B cells. Although a limited diversity of B cell clones has been observed following secondary immunization (42), this result may be an artifact from amplification of bulk DNA (as opposed single cell analysis). However, we feel this is not the case as such limited diversity was not found in DNA amplified in the same fashion from sorted GC populations (although we performed our analysis on memory B cells following either primary or secondary immunization, the limited diversity did not correlate with the number of immunizations). Currently, we are unsure why rearranged λ V regions from β-gal + memory B cells contain such a low clonal diversity. However, the absence of mutated clones derived from β-gal − memory B cells and the significantly reduced (>100-fold) AFC response from transferred β-gal − memory B cells suggests the vast majority of the Ag-specific memory B cell pool is marked by β-gal expression.

Conventional dogma of secondary Ab responses postulates that, upon secondary Ag exposure, AFC differentiation of GC-derived memory B cells constitutes the rapid increase in Ag-specific Ab that is typical of secondary immune responses. The pattern of β-gal expression among secondary AFCs following Ag recall of immune GCCxR26R mice suggests this may not be the case.

Importantly, the hypermutation status of rearranged λ V regions within secondary AFCs showed a strong correlation with β-gal expression. Together, this evidence suggests that the development of β-gal − AFCs following secondary Ag challenge of immune GCCxR26R mice may originate from 1) unmutated, extra-GC memory B cells; or 2) recruitment of virgin B cell clones via immune complex formation. Recent reports have demonstrated the presence of unmutated (IgH chain) NP-specific memory B cells following immunization with NP-conjugated Ag (35–37). Furthermore, these (nonmutated) memory B cells were shown to develop in the absence of GC reactions, suggesting an alternative developmental pathway of memory B cell formation. Our finding of β-gal − unmutated, secondary AFCs is consistent with the concept of extra-GC memory B cell development. Alternatively, it has long been recognized that immunization with soluble Ag in the presence of Ag-specific serum IgG, or with immune complexes generated in vitro, has the ability to enhance the differentiation of primary AFCs in naïve mice (reviewed in Ref. 43). This process can also enhance the formation of GCs and subsequent B cell memory (44–47). Previous experiments using an adoptive transfer model in rats demonstrated the recruitment of virgin B cell clones during anamnestic responses support our findings (48, 49). Although these studies strongly suggest the participation of naïve B cells during memory responses, the contribution of Ab-mediated enhancement of primary AFC differentiation and GC formation during the course of a secondary Ab response has not been thoroughly investigated, due in part to the inability to discriminate between memory and naïve B cells. It is likely that both pathways (i.e., unmutated, extra-GC-derived memory B cells and immune complex formation) contribute to the development of β-gal − secondary AFCs. Finally, the recovery of small numbers of mutated β-gal − secondary AFCs suggests the possibility that not all GC B cells destined for memory were marked by β-gal expression. Further experimentation is underway to elucidate the contribution of each of these pathways has to the development of β-gal − AFCs following Ag recall.
Although it is known that Ag recognition is not an absolute requirement for memory B cell persistence (50), the mechanism underlying the replenishment of B cell memory following Ag recall remains an open question. A likely scenario is one in which self-renewal of memory B cells occurs in addition to AFC production following Ag recognition by memory B cells (51). In addition to β-gal+ APCs, a second population of β-gal− B cells (B220+Synd-1−) also was evident in the spleen of GCCxR26R mice following Ag recall. These cells may represent either 1) self-renewing memory B cells, 2) memory B cells participating in secondary GC, or 3) secondary GC B cells derived from naive B cell clones. The fact that B220−β-gal+ Synd-1− B cells following Ag recall contained mutation frequencies (and patterns) that significantly differed from their B220+β-gal− Synd-1− counterparts suggests that these cells were derived from a population distinct from β-gal+ memory B cells and therefore do not represent a self-renewing memory B cell compartment. The mutation data further imply that secondary GC were not seeded by β-gal+ memory cells, consistent with the notion that further rounds of mutation would be deleterious to BCR previously optimized for Ag binding. In fact, both the mutation frequency and PNA profile of B220−β-gal− Synd-1− B cells following Ag recall (20–30% PNA−; data not shown) were similar to that found during the primary response, and suggests this compartment consisted of nascent, secondary GC B cells derived from virgin B cell clones, or possibly non-GC-derived, unmutated memory B cells. The replenishment of B cell memory may involve the production of B220− memory B cells (52) or may occur in other compartments such as bone marrow. Indeed, we detected β-gal− B220+ as well as β-gal− B220+ cells in bone marrow taken from immune GCCxR26R mice (data not shown). Future experiments will be aimed at analyzing these possibilities for the presence of self-renewing memory B cells following secondary Ag challenge.

An obvious benefit of this model will be the ability to analyze the development of memory B cells and their responses to diverse Ags, including multigene, naked DNA, and live viral vaccines. Vaccination regimens using protein (plus adjuvant) or DNA Ag typically produce weak immunizing responses compared with those using attenuated viral vectors. These outcomes most likely reflect differences in the generation of memory B and T cell compartments, as life-long immunity can only be achieved following secondary and higher-order immune responses.

Acknowledgments
We gratefully acknowledge Andrea Siegal for contributing the RAG2-deficient mice used in these studies, Karin Smith and Lorra Miller for mouse colony management, and the Yerkes National Primate Research Center Microarray Facility (Emory University, Atlanta, GA) for assistance with DNA sequencing. We also are grateful to David Baltimore for critical reading of the manuscript.

Disclosures
The authors have no financial conflict of interest.

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