Preferential localization of IgG memory B cells adjacent to contracted germinal centers

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It has long been presumed that after leaving the germinal centers (GCs), memory B cells colonize the marginal zone or join the recirculating pool. Here we demonstrate the preferential localization of nitrophenol-chicken γ-globulin-induced CD38+ IgG1+ memory B cells adjacent to contracted GCs in the spleen. The memory B cells in this region proliferated after secondary immunization, a response that was abolished by depletion of CD4+ T cells. We also found that these IgG1+ memory B cells could present antigen on their surface, and that this activity was required for their activation. These results implicate this peri-GC region as an important site for survival and reactivation of memory B cells.

Appropriate interactions between antigen-specific B and T lymphocytes are essential for humoral immune responses to T-dependent antigens (1, 2). After their initial exposure to antigen, antigen-binding IgM+ B cells migrate from random locations within the B-cell follicles to the border between the follicles and the T-cell–rich areas, where cognate interactions with antigen-specific CD4 T cells occur and subsequent B-cell proliferation is induced (3, 4). Then, shortly after the appearance of extrafollicular foci of antibody (Ab)-secreting plasma cells, clusters of isotype-switched cells such as IgG+ B cells, which can be detected by staining with peanut agglutinin and the GL7 mAb, appear in germinal centers (GCs) within the areas occupied by follicular dendritic cells (5–7). Because the Ig variable-region genes of IgG+ long-lived memory B cells contain somatic mutations, and because these mutations occur primarily in GCs, it is thought that IgG+ memory B cells are mainly derived from the GC.

Despite the importance of IgG+ memory B cells in long-term humoral immunity (8, 9), their sites of residency and activation remain elusive, in part, because of technical difficulties associated with in situ detection of the rare IgG+ memory B cells specific for a given antigen. Thus, to circumvent this problem, a transgenic mouse line harboring an IgM-type B-cell antigen receptor (BCR) has been used; these studies showed that long-lived IgM+ memory B cells reside not just in the marginal zone (MZ), as had been thought, but also in splenic follicles (10). However, given the finding that antigen-experienced IgM B cells and switched IgG2a B cells differentially localize during primary immune responses (11), extrapolation of the above scenario to residency sites of IgG+ memory B cells in physiological settings needs to be done with great caution.

Here we focus on where IgG+ memory B cells reside and how these memory B cells are activated upon secondary antigen challenge.

Results

CD38+IgG1+ Memory B Cells Localize Around GCs. The Ab response to the hapten nitrophenol (NP) has been characterized extensively (12, 13). Thus, to determine where IgG-type memory B cells reside, we used this model system. The response to NP in C57BL/6 (B6) mice is dominated by Abs composed of the VH186.2 heavy chain and an Igλ light chain (12, 13). Consistent with these reports, by flow cytometry analysis, ∼60–80% of NP-specific IgG1+ B cells expressed λ light chains on day 60 after alum-precipitated NP-conjugated chicken γ-globulin (NP-CCG) immunization (Fig. S1A). We then used immunohistochemical analyses to determine the localization of IgG1+ λ+ B cells, which are mixtures of memory and GC B cells as described below, in the spleen on day 30 and day 60 postimmunization. As shown in Fig. S1B, IgG1+ cells were located in several areas including the red pulps and follicles, but the λ-expressing IgG1+ cells were found mainly as clusters residing in the centers of follicles both at day 30 and day 60. Although we expected considerable effort attempting to detect NP-reactive cells in sections using NP-labeled fluorochromes, in our hands the data obtained using this approach were unconvincing.

Previous studies have demonstrated that CD38, used in conjunction with GL7, is a good marker for distinguishing between memory and GC B cells: The former are CD38+IgG1+ and the latter are CD38+IgG1+GL7+ (14, 15). Therefore, to localize IgG1-type memory B cells, we stained sections of spleen from NP-CCG-immunized mice with anti-CD38, -IgG1, and -GL7 Abs. On day 30, a small number of IgG1+CD38+ B cells were detected in the follicles adjacent to clusters of CD38+GL7+ GC B cells (Fig. 1A). On day 60, these IgG1+CD38+ B cells were detected in the follicles adjacent to clusters of CD38+GL7+ GC B cells (Fig. 1A). On day 60, these IgG1+CD38+ B cells were still observed near the clusters of CD38+GL7+ GC B cells; the GC B cells were still present, despite a significant reduction in their numbers compared with day 30 by both flow cytometry and immunohistology (Fig. 1B). Results using PNA, another widely used marker for GC B cells, were similar to those with the GL7 mAb (Fig. S2). Moreover, consistent with the above results, ∼50–70% of IgG1+ cells clustering near the GCs on day 60 were λ+ (Fig. 1A). Together, these observations suggest that the majority of IgG1+ memory B cells localized near the contracted GCs on day 60 were specific for NP.

IgM- and IgG-type Memory B Cells Localize in Distinct Areas of the Spleen. Several previous reports have described a different localization of memory B cells from the one we describe above, that is, scattered in follicles or within the marginal zones (10, 16).

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Because previous reports mainly focused on memory B cells expressing IgM, we next examined the possibility that IgM- and IgG-type memory B cells might differentially localize in the spleen. For this purpose, we used mice that have a heavy-chain locus targeted with the B1-8hi IgH (Ighhi) gene. Because the light chains in B1-8hi mice are not fixed, only 3–5% of their B cells express λ and bind NP (17). F1 offspring of CD45.1 and CD45.2 congenic mice (CD45.1-CD45.2 F1) were adoptively transferred with B cells from B1-8hi IgH knock-in mice (CD45.2) and immunized with alum-precipitated NP-CGG. In this experimental setting, almost all donor-derived B cells present on day 30 after immunization can be considered as antigen-experienced, because of the following two lines of evidence. On day 30, more than 90% of the transferred B cells bound NP, in contrast to about 5% of naive B cells (Fig. S3). Second, more than 90% of transferred B cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) before transfer became CFSE-negative by day 30 after immunization, indicating that they had undergone extensive proliferation (Fig. S3). As shown in Fig. 2A, two distinct subpopulations of donor-derived B cells were observed in immunized mice on day 60. The largest subpopulation was CD38hiGL7+, the phenotype of memory B cells, and most of these cells expressed IgM. As expected, those IgM+ cells could bind NP, indicating that NP-reactive IgM-type memory B cells are generated in this experimental system. Histological analysis revealed that the IgM-type memory B cells detected with anti-IgM mAb were mainly scattered in the same follicle in different sections. As shown in Fig. 2B, immunohistological (Upper) and immunofluorescence-activated cell sorting (FACS) profiles of an unimmunized B6 mouse (day 0) are also shown. White arrows in the first and second rows of the second section indicate cells stained with both anti-CD38 and -IgG1 Abs, and in the third row indicate cells that are IgG1+. [Scale bars, 150 μm (Left), 50 μm (Center).] (B) Quantification of the absolute number of memory (CD38hiIgG1+) and GC (CD38hiIgG1+) B cells by flow cytometric (Left) and immunohistological (Right) analyses.

Fig. 1. Detection of CD38hiIgG1+ memory B cells adjacent to contracted GCs. (A) B6 mice were immunized with alum-precipitated NP-CGG. After 30 and 60 days, spleens were harvested and subjected to flow cytometric (Upper) and immunohistological (Lower) analyses. B220+ gated populations are shown in the upper panels. A fluorescence-activated cell sorting (FACS) profile of an unimmunized B6 mouse (day 0) is also shown. White arrows in the first and second rows of the second section indicate cells stained with both anti-CD38 and -IgG1 Abs, and in the third row indicate cells that are IgG1+. [Scale bars, 150 μm (Left), 50 μm (Center).] (B) Quantification of the absolute number of memory (CD38hiIgG1+) and GC (CD38hiIgG1+) B cells by flow cytometric (Left) and immunohistological (Right) analyses. 

Fig. 2. IgM- and IgG-type memory B cells differentially localize in the spleen. (A) B cells from B1-8hi IgH knock-in mice were adoptively transferred to CD45.1-CD45.2 F1 mice. The mice were immunized with alum-precipitated NP-CGG or left untreated. After 30 days, unimmunized mice were killed and analyzed for the presence of donor-derived, B220+CD45.1+ cells by flow cytometric analysis (Upper Left; None, day 30). Immunized mice were killed at 60 days after immunization (NP-CGG + Alum, day 60). Spleens were subjected to flow cytometric (Upper) or immunohistological (Lower) analyses. [Scale bars, 300 μm (Left), 20 μm (Right).] (B) B cells purified from double-transgenic mice (B1-8hi IgH knock-in and Fucci-red) were adoptively transferred to CD45.1-CD45.2 F1 mice. The mice were immunized with alum-precipitated NP-CGG or left untreated. After 60 days, spleens from unimmunized (None) or immunized (NP-CGG + Alum, day 60) mice were subjected to flow cytometric (Upper) and immunohistological (Lower) analyses. The panels of histological analysis represent the same region in the same follicle in different sections. [Scale bars, 300 μm (Left), 50 μm (Center), 100 μm (Right).] (C) Quantification of IgM+ cells in A and IgG1+ Fucci-red cells in B are shown. Average number ± SD from three mice are shown. Cells in the red pulp were probably plasma cells, as judged by low expression of CD38. Fo. Scat., IgG1+ cells scattered among follicles.

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transgenic B1-s<sup>hi</sup> IgH knock-in mice, and immunized with NP-CGG precipitated in alum. As shown in Fig. 2B, donor-derived Fucci-red-labeled cells were observed in mice on day 60 after immunization. Flow cytometric analysis revealed that IgG1<sup>+</sup> cells were present in the Fucci-red-labeled cell population. Because we gated out Fucci-red-negative cells, probably including GC B cells, most of these IgG1<sup>+</sup> cells expressed CD38 and were able to bind NP, suggesting that NP-specific IgG1-type memory B cells were labeled with Fucci-red. Localization of Fucci-red-labeled cells was examined by immunohistological analysis (Fig. 2B Lower). Clustering of Fucci-red-labeled CD38<sup>+</sup> cells was observed near GL7<sup>+</sup> CD38<sup>−</sup> GC cells, in addition to cells scattered in follicles. Staining the sections with anti-IgG1 Ab revealed that IgG1<sup>+</sup> cells were predominantly located in clusters near GCs (Fig. 2B and C Right). Together, these data demonstrate differential localization of IgM- and IgG1-type memory B cells.

**Detection of Memory B Cells Based on the AID-Cre-Mediated Expression of Red Fluorescent Protein.** In addition to B cells, follicular dendritic cells are located within and/or surrounding GCs, where they can capture antigen–IgG complexes by virtue of Fce<sub>r</sub> (19) and complement (20) receptors during immune responses. Because the above assay relies on anti-IgG1 staining, one concern is that this method might not be detecting B cells but instead cells that had passively acquired secreted IgG1 Abs. To eliminate this possibility, we used a genetic approach in which the Cre recombinase gene is expressed under the control of the Aicda (activation-induced cytidine deaminase; AID) promoter and red fluorescent protein (RFP) is only expressed upon Cre-mediated deletion of a floxed neomycin gene (AID-cre/RFP-ROSA) (Fig. S4) (21, 22). In these mice, the progeny of AID-expressing cells, including memory B cells, are permanently RFP<sup>+</sup> (Fig. S5A).

Localization of the red fluorescent cells was determined by immunohistological analysis (Fig. S5B). Consistent with the above immunohistological analysis using anti-IgG1 Ab, RFP<sup>+</sup>CD38<sup>−</sup> B cells were also located near the GL7<sup>+</sup> GC B cells (Fig. S5B Left). Most of these RFP<sup>+</sup>CD38<sup>−</sup> B cells were IgG1<sup>+</sup> (Fig. S5B Right). Based on these findings, we conclude that most IgG1 memory B cells in the spleen are found in clusters near the contracted GCs.

**Location of the CD38<sup>+</sup>IgG1<sup>+</sup> Memory B Cells That Proliferate Following Antigen Rechallenge.** To next examine where memory B cells are activated upon secondary antigen challenge (NP-CGG without alum), we used another strain of Fucci transgenic mice, in which cells become reversibly fluorescent (green) in the S/G2/M, but not G1, phases (Fucci-green) (18). As expected, flow cytometric analysis indicated that almost all of the CD38<sup>+</sup>IgG1<sup>+</sup>NP<sup>+</sup> memory B cells on day 60 after primary challenge were in the resting stage. However, by 2 days after secondary challenge, many of them had entered the cell cycle and become green (Fig. 3A). In situ studies were performed to identify the location of the green cells in the spleen (Fig. 3B). Before secondary challenge, a few green B cells were detectable, consistent with the flow cytometric data (Fig. 3A). Two days after secondary challenge, many IgG1 B cells entered the S/G2/M phase. Among these green cells, about two-thirds of them were CD38<sup>−</sup> memory cells and the remaining were CD38<sup>+</sup> GC B cells. These results suggest that IgG1 memory B cells near the contracted GCs, together with the GC B cells, start to proliferate upon secondary challenge.

**CD4<sup>+</sup> T Cells Reside Close to IgG1<sup>+</sup> Memory B Cells in the Follicles.** Considering the recent evidence that some T cells, particularly follicular helper T cells (TFH), are localized inside or surrounding GCs during primary humoral responses (23–25), it seemed possible that helper T cells for activating memory B cells might also reside near the contracted GCs. If so, in contrast to the requirement for migration of naïve B and naïve T cells toward the T-B border area for their initial cognate interactions, such active migration might not necessarily be required for activating memory B cells. This possibility was tested by immunohistological analysis of spleen sections on day 60 after primary immunization. As shown in Fig. 4A, CD4<sup>+</sup> T cells were found near the region where CD38<sup>+</sup>IgG1<sup>+</sup> and CD38<sup>+</sup>IgG1<sup>+</sup> B cells were localized in the follicles. To further examine whether these CD4<sup>+</sup> T cells express TFH markers, we used anti-CD4 mAb (26) and found that some, but not all, CD4<sup>+</sup> T cells in the follicles on day 60 after primary immunization expressed PD-1 (Fig. 4B).

**Cognate Interaction of IgG1<sup>+</sup> Memory B Cells with CD4<sup>+</sup> T Cells Is Required for Their Activation.** The presence of T cells near the IgG1 memory B cells prompted us to examine the functional requirement for such helper T cells in humoral memory responses. To address this question, B6 or Fucci-green transgenic mice that had been immunized with alum-precipitated NP-CGG were treated with anti-CD4 mAb and control Abs before secondary challenge (NP-CGG without alum). The initial proliferation of the memory B cells, as judged by the expression of the Fucci-green probe (Fig. 4C), as well as the production of secondary anti-NP Abs (Fig. 4D) were almost completely abolished by the anti-CD4 treatment.

Having demonstrated the importance of CD4<sup>+</sup> T cells for activating IgG1 memory B cells, we wished to address whether cognate interactions between the B and T cells are required. Before addressing this question, we examined whether the IgG1 memory B cells are capable of presenting antigen. To do this, NP-CGG-primed B6 mice were boosted with an NP-conjugated fusion protein composed of GFP and amino acids 46–74 of the...
I-E\(\alpha\) MHC II subunit (NP-E\(\alpha\)GFP) as a secondary challenge antigen. In this setting, antigen presentation can be monitored with the Y-Ae mAb, which is specific for pE\(\alpha\):I-Ab complexes on the surface of antigen-presenting cells (27, 28). As demonstrated in Fig. 5A, CD38\(^{+}\)IgG1\(^{+}\) memory B cells were able to present exogenous antigens in the context of MHC class II molecules. In these experiments, IgM\(^{+}\) and IgD\(^{+}\) cells were not depleted before flow cytometric analysis for technical reasons, and thus the large number of NP-binding IgG1\(^{-}\) cells is probably IgM\(^{+}\) B cells.

Next, to test the requirement for antigen presentation by memory B cells in their activation, sorted CD38\(^{+}\)IgG1\(^{+}\)NP\(^{+}\) memory B cells were transferred together with CGG-primed CD4\(^{+}\) T cells into Rag\(^{1-/-}\) mice. The memory B cells were able to mount a secondary Ab response when the recipient mice were administered NP-CGG, but not NP-chicken ovalbumin (NP-OVA) (Fig. 5B). The simplest explanation for these results is that IgG1\(^{+}\)NP\(^{+}\) memory B cells efficiently differentiated only when expression of an NP-specific IgG1 BCR allowed efficient uptake of CGG and presentation of CGG peptides to the CGG-primed T cells. NP conjugated with a different carrier, OVA, would be unable to elicit the requisite T-cell help in this system. This idea was substantiated by our findings using MHC class II\(^{-/-}\)Rag\(^{1-/-}\) mice as recipients, an experimental setting in which MHC class II is only expressed on the transferred cells.

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then emerge in both the follicle and the MZ at later time points (16). Although the study using rats did not distinguish whether these memory B cells were of the IgM or class-switched type, given that the same localization sites was observed when NP-specific IgM transgenic mouse models were immunized (10) it is most likely that the NP-specific memory B cells observed in the rat system were of the IgM type. This possibility is further supported by our observations that antigen-experienced IgM+ cells are scattered in the follicles on day 60 after primary immunization (Fig. 2A and C).

In contrast to the localization of IgM-type memory B cells, we have shown here that IgG1-type memory B cells (IgG1+CD38+−) are mainly located near the contracted GC-like structures still present on day 60 after primary immunization (NP-CGG with alum). Our histochemical resolution did not suffice to conclude whether the IgG1+CD38+− memory B cells are localized near the GC light or dark zone. The presence of these GC-like structures on day 60, albeit much smaller than on day 30, is quite consistent with a recent report demonstrating that GC-like structures persist for up to 8 months after being challenged with sheep red blood cells (SRBCs) twice (29). Because SRBCs induce a very potent polyclonal B-cell response, the persistence of GC-like structures for longer periods in the case of SRBCs probably reflects the fact that a steady-state level of newly activated B-cell clones is high, thereby being continuously recruited into the GC fractions. Together with our data, it now seems clear that GC-like structures can persist longer than previously appreciated, and that the duration of such structures is dependent, at least partly, on the nature of the immunogen and adjuvants.

By using lymphocytes harboring the Fucci cell-cycle tracker, we have demonstrated here that IgG1+CD38+− memory B cells near GCs, in addition to the IgG1+CD38+− (GC B cells from our criteria), begin to proliferate upon secondary challenging on day 60. As discussed above, in the NP-CGG immunization protocol, some IgG1+CD38+− GC B cells still remain at day 60 and their proliferation appears to be enhanced upon secondary challenge. Because recent reports have suggested that IgG-type memory B cells undergo differentiation into plasma cells rather than entering into GC pools (29, 30), we favor the idea that the precursors of proliferating IgG1+CD38+− B cells on day 2 after secondary challenge are GC, but not memory, B cells. However, at present, we cannot completely exclude the possibility that IgG1+CD38+− memory B cells differentiate into IgG1+CD38+− cells, or vice versa, during the 2 days after secondary challenge. Assuming that such preferential localization and activation of memory B cells near the contracted GCs also occur in draining lymph nodes, our observations would explain the previous findings that the ipsilateral lymph node transfers a significantly higher humoral memory response than does the contralateral node at all intervals after a primary challenge (31).

Proliferation of IgG1+CD38+− memory B cells and their subsequent differentiation into plasma cells are likely to require cognate interactions between B and T cells. This notion is supported by the following three lines of evidence: (i) the requirement for T cells for the proliferation and differentiation of IgG1 memory B cells; (ii) IgG1+CD38+− memory B cells are able to present antigens; and (iii) the requirement for MHC class II on B cells for differentiation of IgG1+CD38+− memory B cells. Given that CD4+ T cells exist in close proximity to IgG1 memory B cells near the contracted GCs on day 60, we speculate that some of these CD4+ T cells are long-lived follicular helper memory T cells and are responsible for activation of IgG1 memory B cells. If so, we would propose that this close proximity of memory B cells to memory T cells can explain, at least partly, the more rapid kinetics of memory responses because, during primary responses, movement of antigen-specific B cells and antigen-specific T cells toward the T-B border area is required.

Discussion

Previous work has shown that NP-specific memory B cells appear in the MZ area shortly after primary immunization in rats, and could elicit the secondary NP response, whereas administration of Fab fragments of anti-MHC class II mAb blocked this response, demonstrating the importance of MHC class II expression on memory B cells.
In addition to this spatial advantage, intrinsic properties of IgG-type memory B cells likely contribute to their more rapid response as demonstrated in previous studies. For example, IgG1-type memory B cells are more apt to differentiate into plasma cells than IgM-type memory and/or naïve cells (29, 30). At the molecular level, the cytoplasmic domain of the IgM-type BCR is almost nonexistent, whereas the IgG-type BCR contains an extended cytoplasmic domain which has been suggested to generate unique signals for conferring a memory phenotype (32, 33).

Although our study has focused on IgG-type memory B cells, we do not argue that antigen-specific IgM memory B cells are dispensable for T-dependent memory IgG responses. In regard to their contribution, it has been recently proposed that memory IgM B cells, by virtue of their rapid mobilization in GCs and switching to IgG after antigen rechallenge, ensure replenishment of the memory pool, probably including both IgM and IgG types (29).

Our data, together with recent work (29), strongly suggest the importance of persisting GC-like structures for normal humoral memory responses. However, previous studies involving genetic deficiency of CD40 and Bcl6 in human and mouse, respectively (34, 35), demonstrated that in the absence of GCs, an unmutated memory responses colonize the splenic marginal zones. In regard to their importance, persisting GC-like structures for normal humoral memory IgM B cells, by virtue of their rapid mobilization in GCs and switching to IgG after antigen rechallenge, ensure replenishment of the memory pool, probably including both IgM and IgG types (29).

In addition, our data strongly support the role of persisting GC-like structures for normal humoral memory responses. However, previous studies involving genetic deficiency of CD40 and Bcl6 in human and mouse, respectively (34, 35), demonstrated that in the absence of GCs, an unmutated functional memory B-cell compartment could develop, implying that there may exist at least one, if not multiple, GC-independent pathways of memory B-cell development. However, the IgG memory B cells that develop in the absence of Bcl6 appear not to give rise to long-lived plasma cells (34). Thus, it is possible that there are functional differences between GC-dependent and -independent IgG memory B cells.

Methods

Detailed descriptions of all materials and methods are provided in SI Methods.

Mice, Immunization, and Treatment with Anti-Cd4 Monoclonal Antibody. C57Bl/6 mice were purchased from CLEA Japan. B1-8R IgG knock-in mice were described in ref. 17. AID-cre mice and RFP-ROSA mice have been described previously (21, 22). MHC class II-deficient mice (36) were crossed with Rag1<sup>−/−</sup> mice to obtain Rag1<sup>−/−</sup> MHC class II<sup>−/−</sup> mice. Mice were injected i.p. with 100 μg of NP-CCG in 200 μl alum (Thermo Scientific) according to the manufacturer’s instructions. For secondary immunization, mice were injected i.p. with 50 μg of NP-CCG or Eu5GNP in PBS without any adjuvant. For in vivo depletion of CD4<sup>+</sup> cells, mice were injected i.p. with 200 μg of anti-CD4 mAb (clone; YTS191.1) every day for 3 days before rechallenge with NP-CCG. All of the protocols for animal experiments were approved by the RIKEN Animal Research Committee.

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Supporting Information

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SI Methods

Adoptive Transfer Experiments. Splenocytes from B1-8×1 IgH knock-in or double-transgenic mice (B1-8×1 IgH knock-in and Fucci-red) were negatively selected by anti-CD43 magnetic beads with AutoMACS (Miltenyi Biotech). CD43+ cells (2 × 10^7) were transferred i.v. to CD45.1-CD45.2 F1 mice. Twenty-four hours after cell transfer, mice were immunized with 100 μg of nitrophenol-chicken γ-globulin (NP-CGG) precipitated with alum. On day 30 or day 60 after immunization, mice were killed. Spleens from the mice were subjected to flow cytometric or immunohistological analysis.

Flow Cytometric Analysis. Splenocytes were treated with Fc-block (BD Bioscience) for 30 min on ice. Cells were incubated with biotinylated anti-IgM, -IgD, -Thy1.2, -CD3, -Gr-1, -F4/80, -CD5, -TER119, -DX5, and -NK1.1 antibodies (Abs) followed by incubation with streptavidin-coupled magnetic beads (Miltenyi Biotech). Unlabeled cells were collected by AutoMACS and stained with fluorescently labeled Abs. In the adoptive transfer experiments, splenocytes were treated with Fc-block and stained directly with the fluorescently labeled mAbs indicated in each fluorescence-activated cell sorting (FACS) profile. For staining with anti-IgG1 and anti-IgM+ Abs simultaneously, cells were first stained with anti-IgG1, incubated in PBS containing 5% normal mouse serum, and stained with anti-IgM+ and other mAbs. In the experiments for detection of the Eα peptide associated with MHC class II, splenocytes were depleted of CD43− cells and then stained with the biotinylated Y-Ae mAb. Y-Ae− stained cells were incubated with fluorescence-labeled streptavidin. Data were collected using a FACSCantoII (BD Bioscience) and analyzed with DIVA software (BD Bioscience).

Immunohistology. For preparing sections of spleen from C57BL/6 mice, small pieces of spleen were embedded in OCT compound and frozen at −80 °C. Sections (8-μm) were deposited on slides and then fixed in acetone on ice for 10 min. Spleens from Fucci transgenic mice were fixed in 4% paraformaldehyde in PBS for 2 h at 4 °C followed by cryoprotection with 20% sucrose in PBS for 8–12 h. Fixed spleens were processed as above. Sections were incubated in PBS containing 1% BSA, 10% fetal calf serum (FCS), and 10 μg/mL of Fc-block for 30 min at room temperature. Anti-IgG1 Ab staining was first performed at room temperature for 40 min followed by blocking with PBS containing 4% normal mouse sera for 1 h at room temperature. Blocked sections were stained with anti-GL7, -CD38, -PD-1, -CD4, or -γ light-chain Abs labeled with an appropriate fluorochrome for 40 min at room temperature. Images of stained sections were obtained by SP2AOBS (Leica) or BIOREVO (Keyence). Areas of sections were measured using LCS software or ImageJ software, and cell number/mm2 was calculated for each section.

Reconstitution and Immunization of Rag1−/− Mice. B220+NP+ IgG1+CD38+ memory B cells were sorted from B6 mice immunized with 100 μg of alum-precipitated NP-CGG on day 21 as described previously (1). CD4+ T cells were purified from mice immunized with 100 μg of CGG in alum on day 21 by AutoMACS or FACSaria (BD Bioscience). Rag1−/− or Rag2−/− MHC class II−/− mice were i.v. injected with 3 × 103 memory B cells and 4 × 106 CD4+ T cells. After 24 h, mice were injected with 50 μg of NP-CGG or NP-OVA without adjuvant. In some experiments, mice were injected i.v. with 200 μg of Fab fragments of anti-MHC class II mAb on day 0 and day 3 after cell transplantation. Seven days later, sera were collected for measuring the titer of anti-NP IgG1 by ELISA, or spleens were harvested for measuring the number of Ab-forming cells by enzyme-linked immunospot (ELISPOT) assay.

ELISA. MAX-SORP immunoplates (Nunc) were coated with NP1−BSA, which can capture anti-NP Abs with high but not low affinity, followed by blocking with 0.5% BSA in PBS. Serially diluted sera were added to the wells of plates and incubated for 2 h at room temperature. After washing with PBS, horseradish peroxidase-labeled anti-mouse IgG1 Abs were added to the wells and incubated for 2 h at room temperature. TMB substrate (KPL) was added and absorbance at A450 of each well was measured using a microplate reader (Bio-Rad).

ELISPOT Assay. Plates with a cellulose membrane bottom were coated with NP10−BSA. Splenocytes were added to the wells and incubated in RPMI1640 medium supplemented with 10% FCS, 50 μM 2-ME, and 2 mM sodium pyruvate for 5 h at 37 °C under 5% CO2. After washing with PBS with 0.05% Tween 20, goat anti-mouse IgG1 Ab was added to the wells followed by addition of alkaline phosphatase-labeled anti-goat IgG Ab. Spots were visualized by BCIP/NBT substrate (Promega) and counted.

Fig. S1. \(\lambda^+\)IgG1+ cells preferentially localize in the centers of follicles in spleens of NP-CGG-immunized B6 mice. B6 mice were immunized with alum-precipitated NP-CGG. (A) After 60 d, splenocytes were selected with biotinylated mAbs, as described in SI Methods, and stained with anti-B220, anti-CD38, anti-IgG1, and anti-\(\lambda\) Abs and NP-BSA-PE. \(\lambda^+\) cells in indicated cell populations are shown. (B) After 30 or 60 d, sections of spleens were stained with anti-IgG1, anti-\(\lambda\), and anti-CD38 mAbs. Upper panels show the typical histochemical appearance of stained sections. IgG1+ cells in the red pulp are probably plasma cells, as judged by the high expression of IgG1. [Scale bars, 300 \(\mu\)m (Left), 50 \(\mu\)m (Right).] Lower graphs represent quantification of absolute numbers of IgG1+\(\lambda^+\) cells in sections. Average number \(\pm\) SD from three mice are shown. Fo. Scat. and Fo. Clust. represent IgG1+ cells scattered among follicles and clustered at the center of follicles, respectively. T, T cell area; MZ, marginal zone.

Fig. S2. IgG1+CD38+ cells localize near a cluster of PNA+ cells. Spleens were obtained from B6 mice on day 60 after immunization with alum-precipitated NP-CGG. Sections were stained with PNA and anti-IgG1 and anti-CD38 mAbs. Arrows indicate CD38+IgG1+ cells. [Scale bars, 150 \(\mu\)m (Left), 50 \(\mu\)m (Right).]
Fig. S3. Adoptively transferred B1-8<sup>th</sup> KI B cells are efficiently primed by alum-precipitated NP-CGG. B cells from B1-8<sup>th</sup> KI mice were labeled with 20 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37 °C for 10 min. The labeled cells (1 × 10<sup>7</sup> or 2 × 10<sup>5</sup>) were transferred to CD45.1-CD45.2 F1 mice. The mice transferred with 1 × 10<sup>7</sup> cells were left untreated and those receiving 2 × 10<sup>5</sup> cells were immunized with alum-precipitated NP-CGG 24 h after cell transfer. Thirty days later, splenocytes were harvested from the mice and subjected to flow cytometric analysis. The few cell divisions observed in unimmunized mice (Upper) probably represent antigen-independent proliferation of naïve B cells reported previously by van Zelm et al. (1).


Fig. S4. Schematic view of the constructs in transgenic mice that promote red fluorescent protein (RFP) expression in B cells expressing AID. This strategy permanently marks GC B cells as well as all of their progeny. Triangles upstream of the RFP gene indicate loxP sites.
Fig. S5. Antigen-experienced B cells are efficiently labeled with RFP in AID-cre/RFP-ROSA mice. The mice were immunized with alum-precipitated NP-CGG. On day 60 after immunization, splenocytes from the mice were subjected to flow cytometric (A) or immunohistological (B) analyses. (A) One to three percent of B cells were labeled with RFP. Because the RFP+ cell population did not contain any IgG1+ cells, we conclude that almost all IgG1-type memory B cells were labeled with RFP. NP-binding IgG1+ cells were found among the RFP+CD38+ cells, indicating that NP-specific IgG1+ memory B cells were labeled with RFP. Among RFP+ cells, we also observed CD38+GL7+GC cells and CD38+IgM+ cells. The latter population includes NP-binding cells, suggesting that NP-specific IgM-type memory B cells were also labeled with RFP. Collectively, the data indicate that IgG1-type memory B cells, as well as GC B cells and IgM-type memory B cells, become labeled with RFP in this system. (B) Sections of spleens were stained with anti-CD38 mAb together with anti-GL7 (Left) or -IgG1 mAbs (Right). White arrows indicate cells expressing both CD38 and RFP. [Scale bars, 100 μm (Left), 50 μm (Right).]