

T-independent type II immune responses generate memory B cells

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Unlike T-dependent immune responses against protein antigens, T-independent responses against polysaccharides confer long-lasting humoral immunity in the absence of recall responses and are not known to generate memory B cells. Here we report that polysaccharide antigens elicit memory B cells that are phenotypically distinct from those elicited by protein antigens. Furthermore, memory B cell responses against polysaccharides are regulated by antigen-specific immunoglobulin G antibodies. As the generation and regulation of immunologic memory is central to vaccination, our findings help explain the mode of action of the few existing polysaccharide vaccines and provide a rationale for a wider application of polysaccharide-based strategies in vaccination.

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B cell responses are classified as T-dependent (T-D) or T-independent (T-I) based on the requirement for T cell help in antibody production (1). T-D antigens are proteins that are processed and presented on MHC class II molecules for recognition by cognate helper T cells. T-I antigens are divided into type I and type II. The former are mitogenic stimuli such as LPS, CpG, or poly-IC that elicit polyclonal B cell activation via Toll-like receptors, whereas the latter are polysaccharides that engage the B cell receptor and thus induce antigen-specific B cell responses.

T-I type II antigens elicit robust and long-lasting primary antibody responses in mice (2), and polysaccharide vaccines such as Pneumovax and Menomune confer long-term humoral protection in adult humans. However, T-I type II antigens do not elicit a recall response; i.e., a boost in antibody production upon secondary immunization (3–6). Nevertheless, splenocytes from mice immunized with T-I type II antigens can respond to secondary challenge when adoptively transferred into naive irradiated recipients, and injection of immune serum into naive recipients before adoptive transfer suppresses this response (3, 4).

T-D antigens elicit memory B cells, which develop in T-D germinal centers and can be identified by somatic mutations in their Ig loci or by surface expression of secondary Ig isotypes (7, 8). T-I type II antigens stimulate extrafollicular foci of plasma cell production (2) and short-lived presumably abortive T-I ger-

minal centers (9, 10). It is not known whether T-I type II immune responses generate memory B cells. Very low levels of somatic hypermutation (11) and low frequency of switching to secondary Ig isotypes during T-I type II responses hinder the identification of T-I memory B cells using these criteria, and it is widely accepted that memory B cells are derived only from T-D responses (1, 7, 8, 12, 13). Here we show that T-I type II immune responses generate memory B cells whose secondary activation by polysaccharides is stringently regulated by antigen-specific IgG antibodies.

RESULTS AND DISCUSSION

T-I type II immune responses generate memory B cells

Memory B cells are quiescent B cells derived from proliferating antigen-experienced precursors (14). We used an *in vivo* BrdU pulse-chase strategy to test whether a model T-I type II antigen 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll elicits memory B cells. To ensure that the analysis was not confounded by BrdU incorporation into dividing bone marrow B cell precursors, we adoptively transferred allo-type-marked (CD45.1⁺) splenic B cells from B1-8^{high} IgH knock-in mice (15) into naive wild-type recipients before immunization and subsequently analyzed only the transferred population. Recipient mice were immunized with NP-Ficoll and fed BrdU for the duration of the proliferative phase of the T-I type II response (days 1–5; reference 15), after which

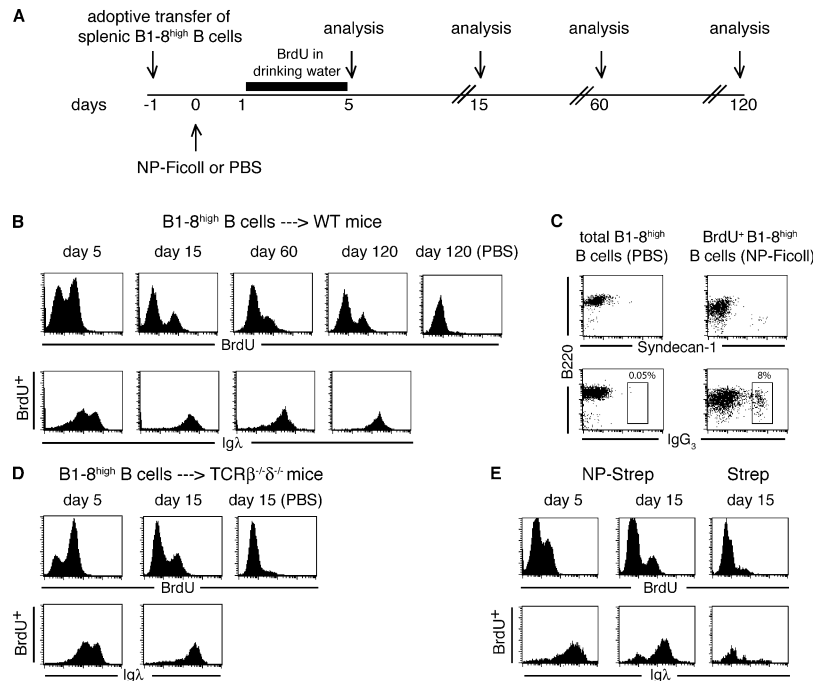


Figure 1. T-I type II immune response generates memory B cells.

(A) BrdU pulse-chase strategy. (B) BrdU staining of adoptively transferred B1-8^{high} B cells (top) and Igλ staining of BrdU-gated B1-8^{high} B cells (bottom) from NP-Ficoll-immunized or naive wild-type recipients analyzed at the indicated time points. (C) B220 versus Syndecan-1 (top) and B220 versus IgG₃ (bottom) staining of B1-8^{high} B cells from naive recipients on day 15 after adoptive transfer (left) and of BrdU-gated B1-8^{high} B cells from NP-Ficoll-immunized recipients on day 15 after adoptive transfer and immunization (right). (D) BrdU staining of adoptively transferred

B1-8^{high} B cells (top) and Igλ staining of BrdU-gated B1-8^{high} B cells (bottom) from NP-Ficoll-immunized or naive TCRβ^{-/-}δ^{-/-} recipients analyzed at the indicated time points. (E) BrdU staining of adoptively transferred B1-8^{high} B cells (top) and Igλ staining of BrdU-gated B1-8^{high} B cells (bottom) from wild-type recipients immunized with NP-coupled (NP-Strep) or noncoupled (Strep) *S. pneumoniae* and analyzed at the indicated time points. All data are representative of two to four independent experiments with two to three mice per time point.

BrdU was withdrawn. Incorporation of BrdU into dividing B cells was assessed by flow cytometry immediately after BrdU withdrawal on day 5 after immunization. To detect quiescent long-term survivors derived from activated precursors, BrdU retention was assayed on days 15, 60, and 120 (Fig. 1 A). We detected allotype-marked BrdU-labeled B cells in the spleen of NP-Ficoll immunized recipients, but not in control recipients injected with PBS and fed BrdU (Fig. 1 B). BrdU-labeled B cells were Igλ⁺ (Fig. 1 B) and thus NP-specific (15, 16). Because subsequent cell division in the absence of BrdU (days 5–120) would have resulted in loss of BrdU by dilution, the detected BrdU-labeled cells must be quiescent.

The BrdU pulse-chase strategy described above might have labeled long-lived terminally differentiated plasma cells and/or quiescent memory B cells. To distinguish between these cell types, we analyzed BrdU-labeled cells for the expression of a plasma cell marker (Syndecan-1) and for antibody secretion. The majority of BrdU-labeled cells expressed B cell marker B220, although at lower levels than naive B cells, and were Syndecan-1⁻ (Fig. 1 C). To verify that BrdU-labeled cells did not secrete antibodies, we purified Igλ⁺ B1-8^{high} B cells by FACS on day 30 after adoptive

transfer and NP-Ficoll immunization and tested them for antibody secretion by ELISPOT. They were indeed nonsecretory (not depicted). Consistent with the possibility that BrdU-labeled cells were memory B cells, 8% expressed a secondary Ig isotype (IgG₃) on their surface (Fig. 1 C). We conclude that quiescent BrdU-labeled B220^{low} Syndecan-1⁻ cells derived from the immune response to NP-Ficoll are memory B cells.

Immune responses against polysaccharides do not require T cells for antibody production (1). To test T cell requirement for the development of memory B cells against polysaccharides, we repeated the BrdU pulse-chase experiment described above in T cell-deficient mice. TCRβ^{-/-}δ^{-/-} mice were adoptively transferred with B1-8^{high} B cells, immunized with NP-Ficoll or PBS, and fed BrdU (days 1–5). To ensure that the transferred B cell sample was not contaminated with T cells, we performed double depletion of CD90.2⁺ cells and confirmed the purity of the sample by CD3 staining (not depicted). BrdU-labeled Igλ⁺ B1-8^{high} memory B cells were detected in TCRβ^{-/-}δ^{-/-} recipients on day 15 after NP-Ficoll immunization (Fig. 1 D). Thus, memory B cell development in response to polysaccharide immunization is T cell-independent.

Pathogens that induce T-I type II responses include bacteria such as *Streptococcus pneumoniae*. We tested whether, similar to NP-Ficoll, epitopes carried on the bacterial cell surface elicit memory B cells. Mice were adoptively transferred with B1-8^{high} B cells, immunized with NP-coupled or noncoupled *S. pneumoniae*, and fed BrdU (days 1–5). The immune response against NP-*S. pneumoniae*, but not against noncoupled bacteria, generated NP-specific (Igλ⁺) memory B cells detected by BrdU staining on day 15 after immunization (Fig. 1 E). Therefore, pathogens that induce T-I type II immune responses stimulate production of memory B cells.

T-I memory B cells resemble naive B cells in their life span and sensitivity to polysaccharide antigens

Naive B cells and T-D memory B cells are long-lived (17–19). To estimate the life span of T-I memory B cells, we compared their survival to that of naive B cells. Adoptively transferred naive Igλ⁺ B1-8^{high} B cells initially constituted 0.27% of total splenocytes, and in the absence of immunization their number declined gradually, reaching half of the initial input in ~90 d (Fig. 2 A). NP-Ficoll immunization resulted in 10-fold expansion of this population by day 5, followed by a sharp decline from 2.7 to 0.7% of total splenocytes between days 5–30 (Fig. 2 A). This loss of Igλ⁺ B1-8^{high} B cells in immunized recipients was disproportionate to the loss of Igλ⁺ B1-8^{high} B cells in naive recipients during the same time period (from 0.27 to 0.22%). After day 30, however, the half-life of the Igλ⁺ B1-8^{high} B cell population was equivalent in immunized and naive recipients. Notably, Igλ⁺ B1-8^{high} B cells remained more numerous in immunized than in naive recipients for up to 4 mo. These results indicate that after the initial expansion and loss of B cells responding to T-I type II antigens, an expanded long-lived population of memory B cells is maintained with a half-life similar to that of naive B cells.

Next, we compared the sensitivity of naive and T-I memory B cells to various doses of a polysaccharide antigen. B cells from naive or NP-Ficoll-primed B1-8^{high} IgH knock-in mice expressing CD45.1 allotype were purified, labeled with CFSE, and adoptively transferred into wild-type recipients. NP-Ficoll priming was performed 30 d before adoptive transfer. The proliferative response of transferred B1-8^{high} B cells was assessed by CFSE dilution on day 5 after NP-Ficoll immunization of recipients. We found no difference between naive and primed Igλ⁺ B1-8^{high} B cells in their responsiveness to doses of NP-Ficoll ranging from 1 to 50 μg (Fig. 2 B). This suggests that naive and T-I memory B cells of the same affinity have similar sensitivity to polysaccharide antigens.

T-I memory B cells differ from T-D memory B cells by cell surface marker phenotype

To determine whether there are phenotypic differences between T-I and T-D memory B cells, we examined their cell surface marker phenotype. B1-8^{high} B cells were adoptively transferred into wild-type recipients, which were then im-

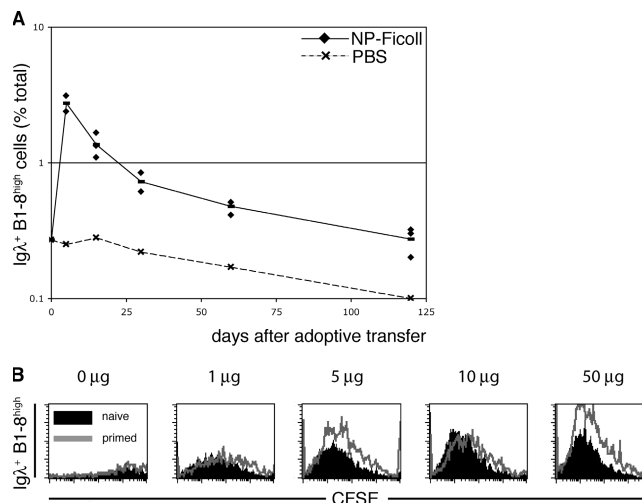


Figure 2. T-I memory B cells resemble naive B cells in their life span and sensitivity to polysaccharide antigens. (A) Survival of adoptively transferred Igλ⁺ B1-8^{high} B cells in NP-Ficoll-immunized and naive wild-type recipients. The number of Igλ⁺ B1-8^{high} B cells per total splenocytes is plotted against the time after adoptive transfer. Average values for each time point are plotted as bars. (B) CFSE dilution by adoptively transferred naive and NP-Ficoll-primed Igλ⁺ B1-8^{high} B cells on day 5 after NP-Ficoll immunization. Doses of NP-Ficoll used for immunization are indicated.

munized either with NP-Ficoll or with a T-D antigen NP-chicken γ globulin (NP-CGG). Igλ⁺ B1-8^{high} B cells derived from T-I or T-D responses differed in the pattern of CD21 versus CD23 expression: the former expressed low levels of CD21 and CD23, whereas the latter resembled follicular (CD21^{low} CD23^{high}) and marginal zone (CD21^{high} CD23^{low}) B cells (Fig. 3 A).

To determine whether such phenotypic differences are found in naturally generated memory B cell populations of T-I or T-D origin, we analyzed the phenotype of memory B cells identified by surface expression of IgG₁+IgG₃ in TCRβ^{-/-}δ^{-/-}, PKCβ^{-/-}, and wild-type mice. IgG⁺ memory B cells in TCRβ^{-/-}δ^{-/-} mice, which can produce only T-I responses, showed the pattern of CD21 versus CD23 expression similar to that of Igλ⁺ B1-8^{high} memory B cells derived from NP-Ficoll immunization (Fig. 3, A and B). In contrast, PKCβ^{-/-} mice, which can produce only T-D responses (20), accumulated IgG⁺ memory B cells resembling Igλ⁺ B1-8^{high} memory B cells derived from NP-CGG immunization (Fig. 3, A and B). Finally, in wild-type mice, IgG⁺ B cells with T-I memory phenotype (CD21^{low} CD23^{low}) constituted ~50% of the total IgG⁺ memory B cell pool (Fig. 3 B). These results indicate that naturally arising T-I memory B cells show the same surface phenotype as Igλ⁺ B1-8^{high} memory B cells that develop in response to NP-Ficoll immunization. Furthermore, T-I memory B cells are phenotypically distinct from conventional T-D memory B cells and comprise a significant portion of the total IgG⁺ memory B cell pool.

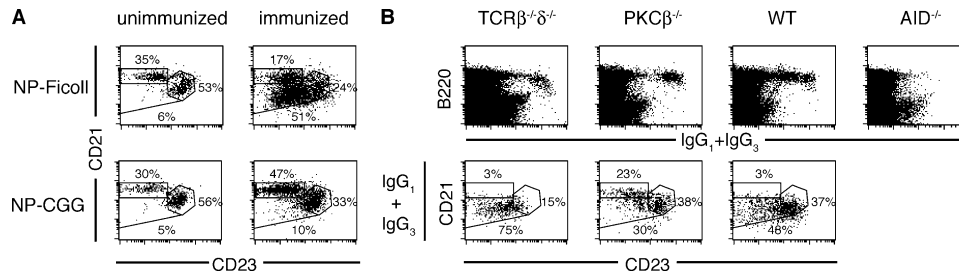


Figure 3. T-I memory B cells are phenotypically distinct from T-D memory B cells. (A) CD21 versus CD23 staining of adoptively transferred Igλ⁺ B1-8^{high} B cells before and after immunization with NP-Ficoll on day 15 (top) or with NP-CGG on day 25 (bottom). (B) B220 versus IgG₁+IgG₃ staining of splenocytes depleted of CD43⁺ and IgM⁺ cells (top) and

CD21 versus CD23 staining of IgG₁+IgG₃-gated B cells (bottom). Mouse strains are indicated. Splenocytes from AID^{-/-} mice, deficient in class switch recombination (reference 21), were used as a negative control for anti-IgG staining.

Secondary activation of T-I memory B cells is regulated by antigen-specific IgG antibodies

Next, we tested whether T-I memory B cells respond to secondary challenge with a polysaccharide antigen. To generate and label T-I memory B cells, wild-type mice were adoptively transferred with allotype-marked (CD45.1⁺) B1-8^{high} B cells, immunized with NP-Ficoll, and fed BrdU (days 1–5). On day 20 after primary immunization, splenocytes were transferred into a second group of naive wild-type recipients and challenged with PBS or NP-Ficoll (Fig. 4 A). Alternatively, BrdU-labeled memory B cells were rechallenged with PBS or NP-Ficoll without adoptive transfer (Fig. 4 C). By day 5 after secondary challenge with NP-Ficoll, but not with PBS, BrdU-labeled memory B cells that had been adoptively transferred into naive recipients lost BrdU due to their proliferation leading to BrdU dilution (Fig. 4 B). In contrast, BrdU-labeled memory B cells that had not been adoptively transferred into naive recipients retained BrdU (Fig. 4 D), thus not responding to secondary challenge with NP-Ficoll. Remarkably, this inhibition of memory B cell proliferation in response to secondary NP-Ficoll challenge was not observed in activation-induced cytidine deaminase (AID)^{-/-} mice (Fig. 4 D) deficient in class switch recombination (21). These results indicate that T-I memory B cells are responsive to secondary challenge with polysaccharide antigens; however, their reactivation is suppressed in primed wild-type mice, but not in AID-deficient or naive mice.

To determine whether the absence of antigen-specific IgG antibodies accounts for the lack of suppression of the T-I memory B cell response, we tested the recall response to NP-Ficoll in AID^{-/-} and wild-type mice. Secondary immunization boosted NP-specific IgM titers in AID-deficient but not in wild-type mice (Fig. 4 E). Next, we injected AID^{-/-} mice with NP-specific IgG antibody, isotype control antibody, or PBS 1 d before secondary NP-Ficoll immunization. NP-specific but not isotype control antibody suppressed the recall response to NP-Ficoll in AID^{-/-} mice (Fig. 4 F). To determine whether IgG-mediated suppression of the T-I type II recall response depends on the inhibitory Fc receptor FcγRIIB (22), we tested the recall response to NP-Ficoll in

FcγRIIB^{-/-} mice. Secondary NP-Ficoll immunization did not boost NP-specific antibody titers in FcγRIIB^{-/-} mice (not depicted). We conclude that antigen-specific IgG antibodies regulate memory B cell responses against polysaccharides via an FcγRIIB-independent mechanism.

We have uncovered an expanded and long-lived population of memory B cells elicited by polysaccharides and bacteria in the absence of T cell help, which differ from conventional T-D memory B cells in their cell surface phenotype (CD21^{low} CD23^{low}). Although more numerous than the original naive antigen-specific B cell pool, T-I memory B cells have a similar life span and sensitivity to limiting doses of polysaccharide antigens compared with naive B cells of the same affinity. Therefore, a distinct characteristic of T-I memory is increased quantity rather than quality of antigen-specific clones, conforming to Burnet's postulate that clonal expansion accounts for immunologic memory (23).

Our data indicate that secondary activation of T-I memory B cells is stringently regulated by antigen-specific IgG antibodies. This regulation is reminiscent of the inhibition of primary humoral responses by passively transferred immune serum, first described by Emil von Behring. This phenomenon is limited to T-I type II and particulate antigens, such as sheep red blood cells (24). It is efficiently mediated by passively transferred antigen-specific IgG antibodies of all isotypes (25) but does not depend on the inhibitory Fc receptor FcγRIIB (26). However, the physiologic relevance of regulation of primary B cell responses by passively transferred immune serum was uncertain because antigen-specific antibodies normally appear only after a productive B cell response. Our findings reveal a physiologic function for IgG-mediated suppression in regulating memory B cell responses to T-I type II antigens.

Because of their poor biodegradability, T-I type II antigens, including synthetic and native bacterial polysaccharides, are retained in the organism for long periods (27). The existence of an expanded and long-lived pool of memory B cells capable of responding to such persistent antigens necessitates a suppressive mechanism to prevent their continuous reactivation leading to antibody overproduction. Antigen-specific

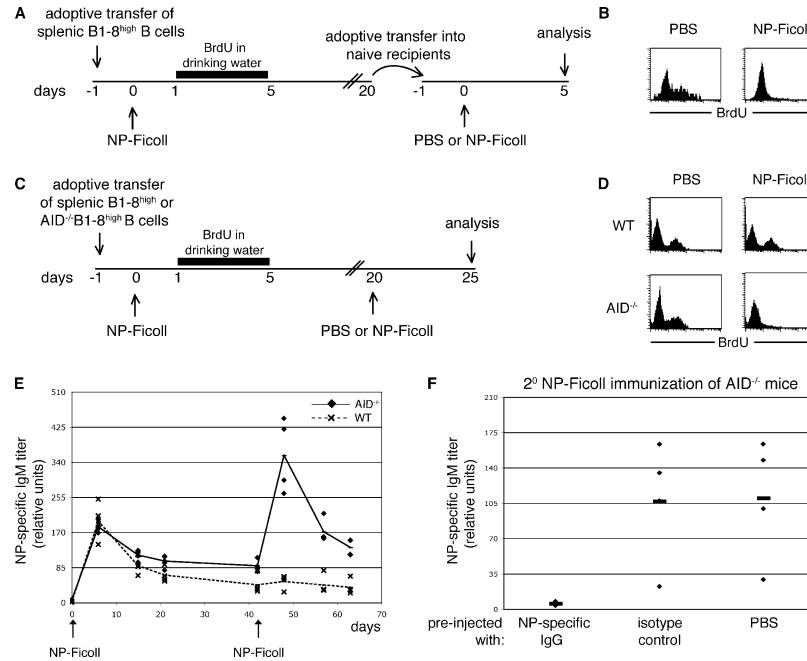


Figure 4. Antigen-specific IgG suppresses T-I memory B cell response. (A and C) Strategies for BrdU labeling and secondary challenge of T-I memory B cells. (B) BrdU staining of Ig λ ⁺ B1-8^{high} memory B cells, generated by NP-Ficoll immunization in the presence of BrdU, after adoptive transfer into naive recipients and secondary challenge with PBS or NP-Ficoll. (D) BrdU staining of B1-8^{high} (top) or AID^{-/-} B1-8^{high} (bottom) B cells adoptively transferred into wild-type or AID^{-/-} recipients, respectively, which were primed with NP-Ficoll in the presence of BrdU and rechallenged with PBS or NP-Ficoll. (E) NP-specific IgM titers versus time after primary and secondary

NP-Ficoll immunization of AID^{-/-} and wild-type mice. Arrows indicate time of immunization. Average values are plotted as bars. Data are representative of three independent experiments with four to five mice per group. (F) NP-specific IgM titers on day 5 after secondary NP-Ficoll immunization of AID^{-/-} mice, preinjected with NP-specific IgG₁, isotype control antibody, or PBS. All mice were primed with NP-Ficoll 1 mo before secondary immunization. Background IgM titer values (before secondary immunization) were subtracted. Average values are plotted as bars. Data are representative of two independent experiments with four to five mice per group.

IgG, a product of the immune response, serves this important negative feedback regulation, thereby maintaining humoral homeostasis. In fact, humans who are unable to produce IgG because of CD40L or AID deficiency suffer from hyper-IgM syndromes (HIGM1 and HIGM2; references 28 and 29).

T-I type II responses have long been thought to lack memory B cell production, which has hindered the effort to develop T-I vaccination strategies. Very few polysaccharide vaccines are currently available in which native bacterial polysaccharide capsule antigens are used. Yet, our data demonstrate the existence of T-I memory B cells and stringent regulation of their secondary activation by IgG antibodies specific to the immunizing antigen. These findings contribute to our understanding of the mode of action of the existing polysaccharide vaccines and argue in favor of a wider application of polysaccharide-based strategies in vaccination.

MATERIALS AND METHODS

Animals and procedures. C57BL/6 and TCR β ^{-/-} δ ^{-/-} mice were purchased from The Jackson Laboratory. B1-8^{high} IgH knock-in mice were generated previously (15). AID^{-/-} mice were provided by T. Honjo (Kyoto University, Kyoto, Japan), PKC β ^{-/-} mice by A. Tarakhovskiy (The Rockefeller University, New York, NY), and Fc γ R1IB^{-/-} mice by J. Ravetch

(The Rockefeller University, New York, NY). All knock-in and knockout strains were on a C57BL/6 background. Mouse procedures were performed under The Rockefeller University's Institutional and Animal Care Use Committee-approved protocols.

Splenic B cells were purified by negative selection with anti-CD43 MACS beads (Miltenyi Biotec). When indicated, an additional round of negative selection was performed with anti-CD90.2 MACS beads. Approximately 2×10^7 purified B cells per recipient mouse were injected i.v. 1 d before immunization. CFSE labeling was performed by incubating B cells (10^7 cells/ml) in PBS containing 5 μ g/ml CFDASE dye for 10 min at 37°C with subsequent PBS washes.

Mice were immunized i.p. with 50 μ g NP₁₉₀-Ficoll (Biosearchtech) in PBS or with 50 μ g NP₁₆-CGG (Biosearchtech) in Imject Alum (Pierce Chemical Co.). *S. pneumoniae* cells (nonlytical Lyt4-4 variant of the strain R36A; provided by A. Tomasz, The Rockefeller University, New York, NY) were grown in C+Y medium (30) until they reached an OD of 0.7 corresponding to a density of 10^8 cells per ml. Bacterial cells were pelleted by centrifugation and incubated for 5 min with 0.5 mg/ml NP-OSu (Biosearchtech). The conjugation reaction was quenched with 1.2 mg/ml glycylglycine in PBS, and the bacteria were washed twice with PBS. Mice were injected i.p. with 10^8 *S. pneumoniae* or NP-coupled *S. pneumoniae* cells. In BrdU pulse-chase experiments, mice were fed BrdU in the drinking water at a concentration of 0.5 mg/ml on days 1–5 after immunization.

The 9T13 hybridoma cell line, provided by T. Azuma (Tokyo University of Science, Noda, Chiba, Japan), was the source of anti-NP IgG₁ antibody. 9T13 antibody was purified from hybridoma supernatants by protein G-Sepharose beads (GE Healthcare) and dialyzed overnight against PBS.

Isotype control antibody (13C4) was purchased from the Rockefeller Monoclonal Antibody facility. 0.4 mg of each antibody was injected i.v. 1 d before secondary NP-Ficoll immunization.

ELISA. NP-specific antibody titers were measured by sandwich ELISA. High-binding plates (Costar) were coated overnight with 5 $\mu\text{g}/\text{ml}$ NP₂-BSA (Biosearchtech), blocked for 1 h with PBS containing 0.2% Tween-20 and 1% BSA, and incubated with serial serum dilutions for 2 h, followed by a 2-h incubation with horseradish peroxidase-conjugated anti-mouse IgM (Jackson ImmunoResearch Laboratories). ELISA plates were developed using 1-Step ABTS (Pierce Chemical Co.). Immune serum from the same mouse was included in all plates, and titers were expressed in arbitrary units relative to that standard.

FACS analysis and BrdU detection. The following antibodies to murine epitopes were used for FACS staining analysis: CD21-FITC, CD23-PE, B220-PE or APC, Syndecan-1-PE, Ig λ -biotin, IgG₁-biotin, IgG₃-biotin, and CD45.1-APC (BD Biosciences). Streptavidin-conjugated PerCP (BD Biosciences) was used as a secondary reagent for biotinylated antibodies. Before staining of rare IgG₁⁺ and IgG₃⁺ B cells, splenocytes were depleted by anti-CD43 and anti-IgM MACS beads (Miltenyi Biotec). BrdU was detected using a BrdU-FITC Flow kit (BD Biosciences).

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