Class-switched memory B cells remodel BCRs within secondary germinal centers

Louise J McHeyzer-Williams1,3, Pierre J Milpied1–3, Shinji L Okitsu1,2 & Michael G McHeyzer-Williams1

Effective vaccines induce high-affinity memory B cells and durable antibody responses through accelerated mechanisms of natural selection. Secondary changes in antibody repertoires after vaccine boosts suggest progressive rediversification of B cell receptors (BCRs), but the underlying mechanisms remain unresolved. Here, the integrated specificity and function of individual memory B cell progeny revealed ongoing evolution of polyclonal antibody specificities through germinal center (GC)-specific transcriptional activity. At the clonal and subclonal levels, single-cell expression of the genes encoding the costimulatory molecule CD83 and the DNA polymerase Polγ segregated the secondary GC transcriptional program into four stages that regulated divergent mechanisms of memory BCR evolution. Our studies demonstrate that vaccine boosts reactivate a cyclic program of GC function in class-switched memory B cells to remodel existing antibody specificities and enhance durable immunological protection.

Antibody-mediated immunological protection relies heavily on the development of high-affinity memory B cells and long-lived plasma cells (PCs). Priming with protein antigens induces antigen-specific follicular helper T cells (Tfh cells) needed to initiate primary-response germinal center (GC) reactions1–3. Following cognate contact, cohorts of antigen-primed B cells form GC microenvironments to expand and diversify germ-line encoded B cell receptors (BCR)3–5. Dynamic imaging has provided a real-time framework for understanding the GC-based evolution of antigen-specific recognition by BCRs6–8. Location-based labeling of GC B cells9, as well as labeled GC Tfh cells10, have provided a new level of understanding of the regulation of GC B cell fate. However, the strict requirement for this spatial organization11, the sequence of GC B cell functions and the dynamics of evolutionary processes that regulate the fate and function of memory B cells during the recall response to antigen remain unclear.

Clonal BCR diversification and the selection of variants of higher affinity are the dominant mechanisms that drive the evolution of antigen-specific B cell memory3–5,12. Somatic hypermutation diversifies antigen-specific BCRs in the progeny of rapidly proliferating GC B cells3,4,12,13. Clonal progeny expressing variant BCRs scan networks of follicular dendritic cells6–8 with varying ability for the uptake, processing and presentation of antigens. In this manner, GC B cells with greater access to antigen make stronger productive contacts with GC Tfh cells14, proliferate more extensively and further diversify the ‘preferred’ and selected antigen-specific BCR15. Containment in GCs and the cyclic progression of BCR diversification can be observed through analysis of the clonal organization of the GC B cell repertoire16,17. However, it remains important to connect these multiple attributes within individual antigen-specific GC B cell clones to understand how specialized GC-specific transcriptional programs drive ongoing BCR rediversification.

Modifying antigen-specific B cell memory at recall is central to antibody-mediated immunological protection. Classic studies have demonstrated a progressive increase in the diversity of memory BCRs during the recall response to antigen18,19. While transfer studies have indicated that memory B cells expand their populations without BCR rediversification20, they have also suggested that ‘selective recruitment’ of affinity-matured memory B cells into PC differentiation might explain ongoing maturation of the antibody repertoire without reinitiation of the GC reaction. Prime-boost studies with protein antigens21,22 and transfer models relying on particular antigens23,24 have reported similar skewing of class-switched memory responses toward PC production. Differences in expression of the transcriptional repressor Bach2 in class-switched memory B cells suggest an intrinsic molecular basis for PC skewing at recall25. In contrast, many studies of circulating human memory B cells analyzing the BCR repertoire26–33 have observed clonal expansion of class-switched memory B cells with BCRs that express shared and unique mutations. Such data suggest an alternative ‘memory BCR-rediversification mode’ that can be used to predict local formation of secondary GCs and ongoing GC function with extended exposure to antigen or a vaccine boost. Subsequent adoptive-transfer studies24,35 have provided supportive evidence for this alternate model, but there remains little insight into local mechanisms.

Here we developed a high-resolution cellular and molecular strategy to monitor the fate of antigen-specific GC B cells in intact primed mice expressing a polyclonal immune system. Our findings demonstrated that the recall response to antigen elicited robust secondary GC reactions in large cohorts of class-switched memory B cells. Secondary GC B cells reinitiated a cyclic GC transcriptional program...
to diversify memory BCR repertoires with ongoing antigen-driven selection at the clonal and subclonal level. Persistent primary GCs were not required for the formation of secondary GCs, and multiple lines of evidence demonstrated that class-switched memory B cells were the main precursor cells in intact primed mice. Our studies identify the local cellular targets and molecular mechanisms that drive ongoing switched-antibody rediversification at recall.

RESULTS
Robust formation of secondary GCs upon antigen recall

Single-cell mapping of the fate of GC B cells among the clonal progeny of memory B cells is a powerful means for monitoring antigen-specific differentiation in vivo. In the absence of clear understanding of the organization and function of memory TFH cells, it is prudent and necessary to assess recall responses without the use of adoptive transfer. Here we used prime-boost immunization with the hapten-protein combination of 4-hydroxy-3-nitrophenylacetyl and keyhole limpet hemocyanin (NP-KLH) to isolate antigen-specific (V_{H}λ1+ NP+) memory-response B cells. After the boost, there was robust emergence of class-switched (IgM-IgD-) antigen-specific GC (GL7−CD38+) B cells that expressed the transcriptional repressor Bcl-6 and small amounts of the homing receptor CD62L (Fig. 1a). Local emergence of these cells after the boost was not dependent on the presence of adjuvant (Fig. 1b). Distinct IgD− GC structures containing networks of CD21−CD35+ follicular dendritic cells, the cytidine deaminase AID, Bcl-6 and GC-localized CD4+ T FH cells were evident in tissue sections by antibody labeling (Fig. 1c). On the basis of flow cytometry and antigen binding, we concluded that carrier protein-specific memory B cells also formed robust switched secondary GCs in draining lymph nodes (LNs) at both time points after the boost (Fig. 1d,e). On the basis of analyses with the same set of markers and cellular dynamics described above, we concluded that changes in priming adjuvant (Supplementary Fig. 1a,b), priming dose (Supplementary Fig. 1c,d) or time after priming (>6 months; data not shown) generated a similar frequency and number of GC B cells after the boost. Therefore, local antigen rechallenge drove robust GC reactions that included large numbers of class-switched antigen-specific GC B cells with broadly similar spatial organization in situ.

Class-switched B cells rediversify BCRs in secondary GCs

To measure ongoing BCR diversification in vivo, we connected the information for phenotype and antibody repertoire for >500 individual switched antigen-specific B cells over the course of the secondary response. Polyclonal B cells that expressed light-chain variable region λ1 and heavy-chain variable region 186.2 (V_{L}λ1-V_{H}186.2) with numerous unique junctional sequences dominated the response in vivo (Supplementary Fig. 2). We used dendrogram displays of near-neighbor sequence alignment to depict the scale of polyclonal relatedness of responding antigen-specific B cells sorted from separate

Figure 1  Class-switched memory B cells form robust secondary GCs. (a) Flow cytometry of class-switched (IgM−IgD−) antigen-specific (NP−λ1+) B cells (CD3−Gr-1− CD19+) in draining LNs before (unimmunized (UI)) and 70 d after priming with NP-KLH (top row), and 4 and 8 d after antigen boost with adjuvant (middle row). Numbers in outlined areas indicate percent NP−λ1+ cells (top row and middle left) or percent GL7−CD38+ cells (bottom right, middle row) (mean ± s.e.m.). Below, expression of Bcl-6 (left) and CD62L (right) by GC B cells (GC) and memory B cells (Mem). (b) Quantification of class-switched antigen-specific B cells (top) and the GC or memory (Mem) subset (bottom) after prime and after boost with (Boost Adj) and without (Boost Sol) adjuvant. Numbers above bars (right) indicate abundance relative to that of memory cells at day 70. ND, not detected. (c) Immunofluorescence of IgD and the follicular dendritic cell marker CD21−CD35, AID, Bcl-6 and the coreceptor CD4 in two serial sections 8 d after boost with soluble antigen. Scale bars, 50 µm. (d) Flow cytometry of B cells (CD3−Gr-1− CD19+ and/or CD138−, IgM−IgD−) in draining LNs of control mice at day 4 after priming with adjuvant only (far left) and in mice at day 8 after boosting with NP-KLH in adjuvant (right), separating KLH-specific and NP-specific B cells (left plots) for further identification as class-switched GC B cells (CD138−B220−GL7−CD38+) in the boosted mice (right plots). Numbers in outlined areas indicate percent KLH-specific cells (top left) or NP-specific B cells (bottom right) (left plots), or percent CD138−B220− cells (left) or GL7−CD38+ cells (right plots). (e) Total class-switched KLH-specific B cells (top) and KLH-specific GC B cells (bottom) in the draining LNs of control mice (unimmunized or at 4 d after immunization with adjuvant only) and mice immunized with NP-KLH at various times (below graph) after prime or boost, with (adj) or without (sol) adjuvant in the boost. Data are representative of five to ten experiments (a,b; mean and s.e.m. of n = 5–10 mice per time point), four experiments (c) or one experiment (d,e; mean and s.e.m. of n = 3 mice per time point in e).
time points after initial priming or the secondary boost. Changes in primary-response GCs, memory B cells before recall and GC B cells after the boost demonstrated large amounts of BCR diversification through polyclonal outgrowth in secondary GC reactions (Fig. 2a). Linear distance from the predicted root of each dendrogram (Fig. 2b), changes in the frequency of mutations per GC B cell (Fig. 2c) and the distribution of changes in amino acids (Fig. 2d) all indicated ongoing memory BCR diversification of class-switched secondary GC B cells. Boost with a soluble antigen induced shorter measured distance to root for the dendrograms (Fig. 2b) and lower numbers of mutations per GC B cell (Fig. 2c) than found with adjuvant. Thus, our single-cell analysis quantified increased BCR diversification in polyclonal switched memory BCR repertoires induced by the vaccine boost and influenced by the addition of exogenous adjuvant.

Transcriptional programs of secondary GC B cells

Imaging strategies have used location to define zonal changes in gene expression that accompany progressive GC B cell function. Here we used high-order 96-gene single-cell quantitative RT-PCR to quantify gene expression in individual antigen-specific B cells (Supplementary Fig. 3). A greater frequency of class-switched secondary GC B cells from day 8 after the boost than memory B cells before the boost expressed genes encoding molecules that serve as cues for guidance and survival in GCs (Ccr5, Ccr4, Baffr and Il21r), indicators of proliferation (Mki67 and Pcsna), molecules involved in BCR diversification (Aicda and Polh), molecules involved in transcriptional regulation (Bcl6, Foxo1, E2a and Id3) and co-modifiers of T cell–B cell contact (Ly75, Slamf1, Cd44a and Fas) (Fig. 2c, top). These indicators of GC-specific transcriptional programming were induced rapidly after the boost in GC B cells, with high frequencies maintained at the single-cell level throughout the first week (Fig. 2c, bottom).

Individual class-switched memory B cells were mostly quiescent and displayed negligible Aicda expression, and there were low frequencies of cells expressing Bcl6 and Mki67 mRNA (Fig. 2f). In contrast, expression of Aicda, Bcl6 and Mki67 mRNA indicated global GC transcriptional activities that persisted 70 d after initial priming, and we also detected this expression on days 4 and 8 after recall in individual secondary GC B cells that had recently undergone population expansion. In the absence of adjuvant at the boost, Aicda mRNA, Bcl6 mRNA and Mki67 mRNA were present at a similar level on a per–GC B cell basis at the same time points following the boost. These data demonstrated reinitiation and ongoing GC-specific transcriptional activities in secondary GC B cells that served to diversify the switched BCR repertoires of polyclonal memory B cells.

GC transcriptional programs assort across four stages

The GC cycle involves sequential transcriptional changes and coordinated cellular function to promote and enhance BCR diversity. To investigate the coordinated programming of multiple progressive GC B cell functions, we calculated the combinatorial associations of gene expression among individual antigen-specific GC B cells.
Figure 3 Expression of Cd83 and Polh assorts four cyclic stages of GC activity. (a) Probability contours of single-cell expression of Cd79b, Bcl6, Aicda, Mki67, Polh, Cd83 and Cxc4 in GC B cells (day 4 and day 8 after a boost as in Fig. 2; n = 372 cells). (b,c) Combination and clustering of the data in a into stages 1–4 in a two-dimensional display by t-SNE (b) and the distribution of the expression of Cd83 and Polh in those stages (c). (d) Distribution of the expression of Cxc4 and Mki67 (used for initial t-SNE clustering) and of Il21r, Slamf1, E2a and Foxo1 based on the t-SNE gates defined in b. (e) Differences in gene expression in successive stages defined by t-SNE, plotted by statistical significance and presented as volcano plots. (f) Heat map of changes in the expression of Cd79b, Bcl6 and select genes with an expression index change of ±2.5 and a P value of <0.05 for that change in at least one of the transitions. Data are representative of three independent experiments.

Principal-component analysis of gene expression by all secondary GC B cells segregated a subset of GC-associated activities into a putative light-zone (LZ) subcompartment (e.g., expression of Cd83, Slamf1, Cd86, Il21r and Myc,) and dark-zone (DZ) subcompartment (e.g., expression of Polh, Cxc4, Mki67 and Cd24a) (Supplementary Figs. 4 and 5). We then used the dimensionality-reduction algorithm t-SNE (‘t-distributed stochastic neighbor embedding’) to identify varied contours of single-cell expression of Polh, Bcl6, Aicda, Mki67, Cd83 and Cxc4 across the secondary GC B cells after a boost as described above (Fig. 3a). With few exceptions, switched GC B cells clustered into four main regions of bivariate t-SNE plots (stages 1–4) representing clustered and segregated transcriptional activity in single GC B cells (Fig. 3b).

Among the selected set of genes, mRNA encoding the costimulatory molecule Cd83 (Cd83 mRNA) and the DNA polymerase Polh (Polh mRNA) contributed most clearly to the clustering of individual GC B cells (Fig. 3c). Given the predicted functions of Cd83 and Polh,41,42 we propose four separate stages of GC transcriptional activity that assort GC B cells to the four regions of the t-SNE plots. Lack of Polh expression suggested no hypermutation machinery, which would place Cd83+Cd83− cells into a LZ compartment designated ‘stage 1’. Increased antigen presentation with potential T cell–B cell contact associated with Cd83 expression would place Cd83+Polh− GC B cells into a separate LZ compartment designated ‘stage 2’. Expression of Polh, indicative of BCR diversification potential in the DZ, would place Cd83−Polh+ GC B cells representing recent arrivals into a DZ compartment designated ‘stage 3’. Loss of Cd83 expression would then place the DZ:Polh− DZ compartment at a greater distance from recent T cell–B cell contact and thus designated ‘stage 4’. Finally, loss of Polh expression with reentry into the LZ before expression of Cd83 would restart the cycle of GC transcriptional programming.

Across the four stages of the proposed GC cycle, the amount of Cxc4 and Mki67 mRNA per GC B cell skewed toward GC cells in the DZ (Fig. 3d, top). A higher proportion of cells in stages 2 and 3 that expressed Il21r and Slamf1 (Fig. 3d, middle), as well as the predicted relationships among cells across the four stages on the basis of coordinated expression of Cd83, Polh, Aicda and Mki67, would support the suggestion of cyclic activity of GC B cells in the proposed model (Supplementary Fig. 6). Furthermore, reentry into the LZ between stages 4 and 1 of the GC cycle was accompanied by decreased expression of Cxc4, Mki67 and Cd24a and increased expression of Cd38 (Fig. 3e, bottom, and f). Antigen presentation and T cell–B cell contact in the LZ between stages 1 and 2 was accompanied by lower expression of Cxc4 and E2a and increased expression of il21r, Slamf1 and Cxc4 and Cxc5 (Fig. 3f, top, and f). Entry into the DZ after T cell–B cell contact between stages 2 and 3 was associated with increased expression of Aicda, Pena, Mki67, Cxc4, Ly75 and Cd24a (Fig. 3e, upper middle, and f). Finally, extended diversification in the DZ between stages 3 and 4 was accompanied by continued high expression of Aicda, Mki67 and Cxc4 and decreased expression of Myc, Ly75 and Il10rb (Fig. 3e, lower middle). These more extensive analyses of coordinated single-cell gene expression were consistent with the proposed cyclic progression of GC B cell transcriptional programming.

Subclonal ‘adaptive radiation’ of switched BCR repertoires
Ongoing selection of diversified antigen-specific BCRs in individual GC B cell clones provides direct evidence of GC function in vivo.16,17.
To investigate the evolutionary dynamics of secondary GC function, we directly connected single-cell gene expression to clonal analysis of the BCR repertoire by integrated single-cell analysis. We performed integrated single-cell analysis of antigen-specific GC B cell clones isolated from individual LNs on days 4 and 8 after the boost (as described above) and defined by unique junctions for

Figure 4 ‘Adaptive radiation’ during subclonal BCR evolution in the GC. (a) Indexed cell sorting of clone G at day 8 after a boost, with individual cells at various stages of the GC cycle (colored symbols) overlaid on contour plots of total populations. (b) Distribution of individual cells from various clones (key) at days 4 and 8 after the boost (with ten or more members isolated) (left) and summary of stage distribution (right). *P < 0.05 (t-test). (c,d) Radial phylograms for clones H and G (c) and summary dendrogram for all clones at day 8 after a boost, showing the stage of GC cycle allocated to each member of the clone based on t-SNE clustering (d). (e) Integrated analysis of protein abundance, presented as mean fluorescence intensity (MFI); mRNA expression (30-Ct value); Vj186.2 mutation pattern (BCR), with quantification of mutations (mut); and t-SNE stage for individual GC cells from clone G at day 8 after a boost (as in a-d). Data are representative of four independent experiments with nine clones and 153 single cells (mean and s.e.m. in b).
complementarity-determining region 3 (using clones with over ten cells per clone; \(n = 9\) clones). The index-sort analysis of the clones indicated that the level of antigen binding, light-chain expression and surface phenotype distribution was similar to that of the total population of GC B cells (Fig. 4a). Furthermore, each clone included subclonal members distributed across all four stages of the GC cycle (Fig. 4b). Fewer subclonal members were in stages 2 and 3 of the GC cycle than in the other stages (Fig. 4b), consistent with the loss of unselected LZ GC B cell variants and exit from the GC after T cell–B cell contact.

The subclonal distribution of BCR mutations reveals GC evolutionary dynamics. We next assessed whether individual GC clones converged toward a single variant on a subclonal branch or diverged across multiple options to progress along diversification in many branches independently. Sequence alignment and near-neighbor analysis of clonal BCR repertoires displayed in radial phylograms indicated subclonal GC B cell activity (Fig. 4c). Given these related distributions of mutational variants, there was evidence of multiple divergent branches of subclonal variants that had reexpanded after selection and had rediversified into separate clusters of GC clonal progeny (Fig. 4c). The separate subclonal clusters also included individual members of the clone that distributed across multiple different GC transcriptional stages (as defined by the t-SNE grouping of four stages described above) (Fig. 4d). In a separate representation of clonal data, we tabulated multiple facets of cellular and molecular secondary GC B cell activity independently of BCR sequence alignment to highlight similar and unique GC programs at the subclonal level (Fig. 4e).

Together the results reported above demonstrated rediversification of the mutated and class-switched BCR repertoire in antigen-specific secondary GC B cells. These data also indicated that antigen drove the selection of subclonal BCR variants within secondary GCs. Furthermore, the segregation of individual GC B cells across the four stages of the GC cycle in separate intraclonal clusters indicated the permissive, ongoing and divergent nature of antigen-specific secondary GC B cell evolution. These results would favor the proposal of a divergent mechanism of evolution and revealed the dynamics of subclonal ‘adaptive radiation’ in secondary GCs for switched memory BCR repertoires.

**De novo secondary GC formation**

GC reactions in LNs draining the immunization site can persist at low levels for extended periods43, and activated B cells can join...
pre-existing GCs. To investigate whether secondary GC reactions can arise anew following the antigen-recall regimes described above, we focused analysis on remote cervical LNs that contained no evidence of persistent primary GCs. In such primed mice, an intravenous boost of antigen without adjuvant induced an equivalent frequency of antigen-specific GC B cells in local LNs draining the initial priming site and remote LNs (Fig. 5a). Next, through the use of blocking antibody to the ligand for the costimulatory receptor CD40 (CD40L), we ablated preexisting GCs in mice primed in a similar way and found that an intravenous antigen boost produced a frequency of GC B cells equivalent to that of untreated control mice (Fig. 5b). In both sets of experiments, antigen-specific memory B cells were the most likely precursor cells in the local secondary GC response at recall. Therefore, the vaccine boost was able to induce new GC reactions, even at lower local levels of antigen, and did not require adjuvant or the presence of persistent primary-response GC reactions.

Class-switched memory B cells can form GCs after transfer

We next used adoptive-transfer models to investigate the origins of the precursor cells in secondary GCs. At day 70 after priming with NP-KLH, we isolated class-switched memory B cells (IgM-IgD-CD19+GL7+) without using antigen specificity and transferred those cells, together with CD4+CD44hi T cells (as a source of memory helper T cells), into immunodeficient recipient mice (of the Rag1<sup>−/−</sup>λ<sup>−/−</sup>λ<sup>−/−</sup> strain). At day 14 after recall, we observed large numbers of non-antigen-specific CD38<sup>−</sup>GL7<sup>+</sup> GCs in the spleen of recipient mice; however, the antigen-specific (V<sub>λ</sub>4<sup>+</sup> NP) GC response (CD38<sup>−</sup>GL7<sup>+</sup>) was variable (data not shown). To overcome such variability within the antigen-specific compartment, we included naive, non-specific B cells (from MD4 mice, which have transgenic expression of a BCR specific for hen egg lysozyme) in the mixture of cells transferred in the procedure described above. This non-specific ‘filler’ cell effect resulted in antigen-specific class-switched memory B cells that consistently produced secondary GC responses at recall (Supplementary Fig. 7a–c).

To investigate memory function under more physiological conditions than transfer into the immunodeficient recipient mice noted above (Supplementary Fig. 7a), we transferred 3 × 10<sup>3</sup> to 5 × 10<sup>3</sup> NP<sup>+</sup> class-switched memory B cells (IgM-IgD-CD138<sup>−</sup>CD220<sup>−</sup>CD19<sup>+</sup>NP+) into naive syngeneic wild-type recipient mice and observed donor-derived Bcl-6-expressing GC B cells in the spleen of recipient mice 7 d after transfer and challenge (Fig. 6a). As expected, isolated CD38<sup>−</sup>GL7<sup>+</sup> antigen-specific GC B cells transferred in this model were not recovered and did not respond to immunization (data not shown). In a third model, we transferred NP<sup>+</sup> class-switched memory B cells into unconditioned MD4 mice (as recipients) and observed a substantial donor-derived Bc1<sup>+</sup> GC B cell compartment after boost (Fig. 6b). Thus, under all conditions tested, class-switched memory B cells displayed the potential to form secondary GC reactions during the recall response to antigen.

IgM<sup>+</sup> memory B cells can form GCs after being transferred and then reactivated with repetitive particulate antigens. Transferring equivalent numbers (3 × 10<sup>3</sup> to 5 × 10<sup>3</sup>) of NP<sup>+</sup>IgM<sup>+</sup> B cells or class-switched memory B cells into unconditioned wild-type recipient mice produced similar numbers of donor-derived memory B cells (CD138<sup>−</sup>CD38<sup>−</sup>GL7<sup>−</sup>) in the spleen of recipient mice at day 7 in each transfer condition (Fig. 6a). Consistent with published findings, a minority of transferred memory B cells lost expression of immunoglobulin M (IgM) after recall (Fig. 6a). Furthermore, NP<sup>+</sup>IgM<sup>+</sup> memory B cells produced tenfold fewer GC B cells than did their class-switched memory counterparts in similar unconditioned recipient mice (Fig. 6a). Therefore, some IgM<sup>+</sup> memory B cells were able to form secondary GCs, but class-switched memory B cells had the dominant precursor potential for the formation of secondary GCs in this model.

Secondary GC precursors are class switched

We next assessed the dynamics and diversity of the antigen-specific IgM<sup>+</sup> B cell recall response to antigen in the intact primed mouse.
The adjuvant monophosphoryl lipid A induced large amounts of class switching to IgG2a and IgG2b but also promoted NP+V_{\lambda}1 T IgM+ memory B cells (Fig. 7a). However, as indicated by Bcl-6 expression, very few of those antigen-specific IgM+ memory B cells resided within persistent primary-response GCs in the LNs at day 70 after priming and before recall (Fig. 7a). Furthermore, V_{\gamma}186.2 IgM+ BCRs from those IgM+ memory B cells were largely unmutated or expressed related but unmutated genes other than the gene encoding V_{\gamma}186.2 (data not shown). The low mutation rate of IgM+ memory B cells was consistent with a GC-independent pathway for this unwrapped memory compartment45,46. After recall, antigen-binding V_{\gamma}1 T IgM+ memory B cell populations expanded predominantly into the antigen-specific CD138+ memory-response PC compartment, while <2% of the total antigen-specific cells had the GL7hiCD38lo GC phenotype (Fig. 7b). Thus, as seen in the adoptive-transfer experiments, antigen-specific IgM+ memory B cells had little secondary GC-forming potential in the intact primed mouse.

At the molecular level, class-switch recombination leaves switch circle transcripts within individual cells that have recently undergone recombination activity. To estimate the degree of class-switch recombination that might occur at recall, we quantified the frequency of circle transcripts that result from the IgM-to-IgG2b immunoglobulin class switch47 in 30-cell samples of antigen-specific IgG2b+ B cells. We detected few samples with such transcripts (10–15%) at either day 3 of the early recall response among GL7+CD38+ IgG2b-switched antigen-specific B cells or by day 5 after recall among either the GL7+CD38+ compartment or the GL7hiCD38+ GC compartment of the draining LNs (Fig. 7c–e). Attesting to the sensitivity of this approach, >95% of GL7+CD38+ IgG2b-expressing B cell samples from early in the primary response showed IgM-to-IgG2b class-switch recombination activity (Fig. 7c–e). Collectively, these results suggested that preexisting class-switched memory B cells, not IgM+ memory B cells, were the dominant antigen-specific precursor cells of secondary GC reactions.

DISCUSSION

In these studies, we developed a novel and high-fidelity means for connecting cellular phenotype, gene expression and BCR repertoire for the individual progeny of antigen-specific memory B cells after the recall response to antigen. Our findings have established the ability of responding memory B cell to rediversify expressed antigen-specific BCRs in secondary GC reactions. In this manner, ‘memory BCR rediversification’ can contribute substantially to the evolution of circulating antibody repertoires. The expression of C\(\delta\)83 and Pol\(\iota\) segregate individual class-switched memory GC B cells across four stages of GC function at the clonal and subclonal level. The assortment and progress of subclonal GC B cell activity indicated divergent adaptive evolutionary dynamics that simultaneously enhanced antigen binding and drove intraclonal diversity at the boost.

At the single-cell level, switched memory BCR rediversification is supported by GC-specific transcriptional programs and is driven by antigen-specific selection9,11,14,15. The primary-response GC microenvironment and GC-specific transcriptional programs are required for the induction of high-affinity class-switched B cell memory and are central to antibody-based immunological protection1–5,12. Here we probed the secondary GC transcriptional program at the single-cell level and found individual class-switched GC B cells in four main clusters. Notably, segregation of the expression of C\(\delta\)83 and Pol\(\iota\) in single cells could be used to predict the cyclic progression of GC function.

CD83 is associated with antigen presentation and the stabilization of major histocompatibility complex class II (ref. 42), and both CD83 protein and C\(\delta\)83 mRNA are upregulated in LZ GC B cells3,9. Differences in the uptake and presentation of antigen and upregulation of molecules at the T cell–B cell interface by GC B cells in the LZ at stages 1 and 2 might facilitate antigen-specific selection. LZ B cells of higher affinity might ‘preferentially’ form longer productive contacts with GC T\(\text{FH}\) cells14, increase their expression of the cell-cycle regulator c-Myc48,49, reenter the DZ9 and undergo more rounds of division15. However, static analysis cannot resolve the time of recruitment, and phenotype-based isolation of cells cannot resolve the dynamics of reentry into the cell cycle. Pol\(\iota\) is an error-prone DNA polymerase41 that is upregulated in DZ GC B cells9 to introduce point mutations in the gene encoding the expressed BCR. We found substantial fractions of individual Pol\(\iota\)-expressing GC B cells that were still expressing C\(\delta\)83 mRNA. This C\(\delta\)83+Pol\(\iota\)+ GC B cell subset upregulated their expression of Aicda, Penca and Mki67, which encode products associated with somatic hypermutation and proliferation. Increased expression of the gene encoding the chemokine receptor CXCR4 and decreased expression of the gene encoding the chemokine receptor CXCR5 serve to relocate these cells to the GC DZ11 (stage 3). Extended DZ-related activity of C\(\delta\)83+Pol\(\iota\)+ GC B cells (stage 4) involved decreased expression of M\(\text{yc}\) and Penca with continued high expression of Mki67 and Cxcr4. Hence, distinct changes in the expression of C\(\delta\)83 and Pol\(\iota\) at the single-cell level may further distinguish reentry into the DZ from ongoing BCR rediversification as components of separable programs of DZ GC B cells.

We have demonstrated that under conditions of local protein vaccination and recall response to antigen, class-switched memory B cells were able to form robust secondary GC reactions. These findings were consistent with early reports of substantial non-PC antigen-specific population expansion36,37 and the emergence of GL7+ GC B cells in response to recall38. Particulate antigens with intravenous priming23,24 engage splenic B cells by potentially different mechanisms, with varying requirements for cognate T\(\text{FH}\) cells and the induction of memory helper T cells. An altered balance of memory B cell subsets35 might also affect the function of transferred memory B cells. Preimmune repertoires of antigen-specific responses of B cells and helper T cells to any foreign antigen will affect the initial priming and potentially alter the recall response. In this manner, intact memory responses to phycoerythrin21,23 may be sensitive to initial priming and adjuvant for the formation of memory helper T cell and B cell compartments. Nevertheless, even after adoptive transfer there is evidence of the formation of secondary GCs by class-switched memory B cells22,25,35, albeit at very low levels compared with the production of PCs. Population-based studies focused on changes in somatic hypermutation after transfer and recall34 also support the proposal of ongoing diversification of switched BCRs.

Modifying the class-switched memory B cell fate at recall provides a powerful opportunity for optimizing circulating antibody repertoires toward enhanced antigen binding. Skewing toward PC formation at recall has been attributed to transcriptional differences in class-switched B cells versus IgM+ naive B cells25. This skewing has also been seen in phycoerythrin-specific recall21 responses to virus-like particles24 and after recall in response to adoptive transfer of sheep red blood cells23. Rapid PC skewing in early splenic recall responses has been observed36,38. Some of these differences can be attributed to the skewing of the vaccine adjuvant toward different memory B cell subsets35. We also emphasize that memory B cell responses to most protein antigens require help from cognate T cells50. Hence, we
predict it should be plausible and advantageous to reformulate the boost adjuvant to favor the secondary GC B cell fate and promote optimal binding of switched antibody repertoires for effective long-lasting antigen-specific immunity.

We propose that complex viral proteins at infection26,27,29,30,32 or in vaccine prime-boost formulations28,31,33 drive similar local BCR-rediversification mechanisms. Hence, the ongoing changes to circulating antibody repertoires reported for many human infectious responses are most simply explained by the induction of local secondary GC reactions. Clusters of recently diversified post-secondary GC memory B cells and PCs in the circulation would be responsible for the ongoing clonal diversification observed in these human studies. By targeting minimal epitopes on complex immunogens, we have revealed divergent evolutionary dynamics at the polyclonal, clonal and subclonal levels in intact primed mice. At the population level, the BCR changes induced converge toward antigen binding of higher affinity3,4,12. However, at the single-cell level, the reinforcement and expansion of multiple subclonal variants indicates a more divergent evolutionary process. Under these conditions, recall responses to variant antigens would permit and then enhance refocusing of memory BCs to antigenic variants of the original vaccine. These powerful evolutionary mechanisms can rapidly remodel antibody repertoires of preexisting memory B cells to optimize antibody-based immunological protection. We propose that targeting class-switched memory B cells to reinitiate GC-specific transcriptional programs would enhance the depth and increase the breadth of vaccine-induced immunological protection.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

Supported by the Foundation Bettencourt-Schueller (P.L.M.), the Swiss National Science Foundation (S.L.O.), the Novartis Jubilaumsstiftung (S.L.O.), the Roche Research Foundation (S.L.O.) and the US National Institutes of Health (AI047231, AI040215 and AI071182 to M.G.M.-W.). This is The Scripps Research Institute manuscript number 26086.

AUTHOR CONTRIBUTIONS

L.J.M.-W., P.L.M. and M.G.M.-W. designed and performed experiments, analyzed the data and wrote the paper; and S.L.O. designed and performed the experiments in Supplementary Figure 1c.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Crotty, S. Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. 29, 621–663 (2011).
2. Fazilleau, N., Mark, L., McHeyzer-Williams, L.J. & McHeyzer-Williams, M.G. Follicular helper T cells: lineage and location. Immunity 30, 324–335 (2009).
3. Victoria, G.D. & Nussenzweig, M.C. Germinal centers. Annu. Rev. Immunol. 30, 429–457 (2012).
4. MacLennan, I.C. Germinal centers. Annu. Rev. Immunol. 12, 117–139 (1994).
5. McHeyzer-Williams, M., Okitsu, S., Wang, N. & McHeyzer-Williams, L. Molecular programming of B cell memory. Nat. Rev. Immunol. 12, 24–34 (2012).
6. Allen, C.D., Okada, T., Tang, H.L. & Oyster, J.G. Imaging of germinal center selection events during affinity maturation. Science 315, 528–531 (2007).
7. Hauser, A.E. et al. Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns. Immunity 26, 655–667 (2007).
8. Schwickert, T.A. et al. In vivo imaging of germinal centres reveals a dynamic open structure. Nature 446, 83–87 (2007).
9. Victora, G.D. et al. Germline center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell 143, 592–605 (2010).
10. Shulman, Z. et al. Follicular helper T cell dynamics in germinal centers. Science 341, 673–677 (2013).
11. Bannard, O. et al. Germline center centroblasts transition to a centrocyte phenotype according to a timed program and depend on the dark zone for effective selection. Immunity 35, 912–924 (2013).
12. Rajewsky, K. Clonal selection and learning in the antibody system. Nature 381, 751–758 (1996).
13. Muramatsu, M. et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AIN), a potential RNA editing enzyme. Cell 102, 553–563 (2000).
14. Shulman, Z. et al. Dynamic signaling by T follicular helper cells during germinal center B cell selection. Science 345, 1058–1062 (2014).
15. Gitlin, A.D., Shulman, Z. & Nussenzweig, M.C. Clonal selection in the germinal centre by regulated proliferation and hypermutation. Nature 509, 637–640 (2014).
16. Berek, C., Berger, A. & Apel, M. Maturation of the immune response in germinal centers. Cell 67, 1121–1129 (1991).
17. Lefrançois, L., Kelsall, R. & Weiss, U. Intracanal generation of antibody mutants in germinal centres. Nature 354, 389–392 (1991).
18. Allen, D. et al. Timing, genetic requirements and functional consequences of somatic hypermutation during B-cell development. Immunol. Rev. 96, 5–22 (1987).
19. Benoist, C. & Milstein, C. Mutation drift and repertoire shift in the maturation of the antibody response. Immunol. Rev. 96, 23–41 (1987).
20. Siekewitz, M., Kocks, C., Rajewsky, K. & Dildrop, R. Analysis of somatic mutation and class switching in naïve and memory B cells generating adaptive primary and secondary responses. Cell 68, 757–763 (1987).
21. Benson, M.J. et al. Distinction of the memory B cell response to cognate antigen versus bystander inflammatory signals. J. Exp. Med. 206, 2013–2025 (2009).
22. Pape, K.A., Taylor, J.J., Maui, R.W., Gearhart, P.J. & Jenkins, M.K. Different B cell populations mediate early and late memory during an endogenous immune response. Science 331, 1203–1207 (2011).
23. Hogan, I. et al. Multiple layers of B cell memory with different effector functions. Nat. Immunol. 10, 1292–1299 (2009).
24. Zabel, F. et al. Viral particles drive rapid differentiation of memory B cells into secondary plasma cells producing increased levels of antibodies. J. Immunol. 192, 5499–5508 (2014).
25. Kompetani, K. et al. Repression of the transcription factor Bach2 contributes to predisposition of IgG1 memory B cells toward plasma cell differentiation. Immunity 39, 136–147 (2013).
26. Corti, D. & Lanzavecchia, A. Broadly neutralizing antiviral antibodies. Annu. Rev. Immunol. 31, 705–742 (2013).
27. Gao, F. et al. Cooperation of B cell lineages in induction of HIV-1 broadly neutralizing antibodies. Cell 158, 481–491 (2014).
28. Jiang, N. et al. Lineage structure of the human antibody repertoire in response to influenza vaccination. Sci. Transl. Med. 5, 171ra119 (2013).
29. Klein, F. et al. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. Cell 153, 126–138 (2013).
30. Liao, H.X. et al. Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. Nature 496, 469–476 (2013).
31. Vollmer, C., Cit, R.V., Weinstei, I.A., Dekker, C.L. & Quake, S.R. Genetic measurement of memory B cell recall using antibody repertoire sequencing. Proc. Natl. Acad. Sci. USA 110, 13463–13468 (2013).
32. West, A.P. et al. Structural insights on the role of antibodies in HIV-1 vaccine and therapy. Cell 156, 633–648 (2014).
33. Wrammet, J. et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature 453, 667–671 (2008).
34. Kaji, T. et al. Both mutated and unmutated memory B cells accumulate mutations in the course of the secondary response and develop a new antibody repertoire optimally adapted to the secondary stimulus. Int. Immunol. 25, 683–695 (2013).
35. Zuccarino-Catania, G.V. et al. CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype. Nat. Immunol. 15, 631–637 (2014).
36. McHeyzer-Williams, L.J., Cool, M. & McHeyzer-Williams, M.G. Antigen-specific B cell memory: expression and replenishment of a novel b220(-) memory B cell compartment. J. Exp. Med. 191, 1149–1166 (2000).
37. McHeyzer-Williams, M.G., Nossal, G.J. & Lalor, P.A. Molecular characterization of single memory B cells. Nature 350, 502–505 (1991).
38. Shapiro-Shelie, M. et al. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. Immunity 19, 607–620 (2003).
39. Huson, D.H. & Scornavacca, C. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Syst. Biol. 61, 1061–1067 (2012).
40. Amir et al, A.D. et al. vSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nat. Biotechnol. 31, 545–552 (2013).
41. Delbos, F., Aoufouchi, S., Faili, A., Weill, J.C. & Reynaud, C.A. DNA polymerase eta is the sole contributor of A/T modifications during immunoglobulin gene hypermutation in the mouse. J. Exp. Med. 204, 17–23 (2007).
42. Tze, L.E. et al. CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven March1-mediated ubiquitination and degradation. J. Exp. Med. 208, 149–165 (2011).
43. Kasturi, S.P. et al. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* **470**, 543–547 (2011).

44. Schwickert, T.A., Alabyev, B., Manser, T. & Nussenzweig, M.C. Germinal center reutilization by newly activated B cells. *J. Exp. Med.* **206**, 2907–2914 (2009).

45. Kaji, T. et al. Distinct cellular pathways select germline-encoded and somatically mutated antibodies into immunological memory. *J. Exp. Med.* **209**, 2079–2097 (2012).

46. Taylor, J.J., Pape, K.A. & Jenkins, M.K. A germinal center-independent pathway generates unswitched memory B cells early in the primary response. *J. Exp. Med.* **209**, 597–606 (2012).

47. Kinoshita, K., Harigai, M., Fagarasan, S., Muramatsu, M. & Honjo, T. A hallmark of active class switch recombination: transcripts directed by I promoters on looped-out circular DNAs. *Proc. Natl. Acad. Sci. USA* **98**, 12620–12623 (2001).

48. Calado, D.P. et al. The cell-cycle regulator c-Myc is essential for the formation and maintenance of germinal centers. *Nat. Immunol.* **13**, 1092–1100 (2012).

49. Dominguez-Sola, D. et al. The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry. *Nat. Immunol.* **13**, 1083–1091 (2012).

50. Aiba, Y. et al. Preferential localization of IgG memory B cells adjacent to contracted germinal centers. *Proc. Natl. Acad. Sci. USA* **107**, 12192–12197 (2010).
ONLINE METHODS

Mice. B6 (C57BL/6J), B6.CD45.1 (B6.SJL-Ptprc<sup>+</sup>Pepc<sup>+</sup>BoyJ), B6.Rag1<sup>−/−</sup> (B6.129S7−Rag1<sup>tm1Mom</sup>/J) and B6.MD4 (C57BL/6-Tg(ghelMD4/4Cgc)/J) mice were bred and housed in pathogen-free conditions at The Scripps Research Institute. The Scripps Research Institutional Animal Care and Use Committee approved all experiments.

Immunizations and antibody treatments. Mice were given primary immunization subcutaneously at the base of the tail with 400 µg NP-KLH (4-hydroxy-3-nitrophenacyl (Biosearch) conjugated to keyhole limpet hemocyanin (Pierce)) mixed with adjuvant based on monophosphoryl lipid A (MPL). Secondary immunizations (boosts) were subcutaneous or intra-venous injection of 100 µg NP-KLH in the absence (PBS only; ‘soluble’) or presence of MPL-based adjuvant administered more than 70 d after priming. Immunization with alum adjuvant (aluminum potassium sulfate; Sigma) precipitated with 1 M potassium hydroxide (Sigma)) and complete Freund’s adjuvant (Sigma) was used where appropriate.

For the ablation of persisting GCs in primed mice, mice were given injection of blocking antibody to CD40L (MR1; BioXCell) three times every other day (300 µg per intraperitoneal injection) starting on week 9 after priming. Mice were killed or were given a boost 6 d after the final injection. Matching doses of polyclonal hamster IgG (31246; Pierce) were used as control.

Flow cytometry. Single-cell suspensions of draining (inguinal and periaort- tic) LNs, nondraining (cervical) LNs and spleen were prepared, followed by incubation in 0.17 M NaN<sub>4</sub>Cl for lysis of red blood cells, then cells were then counted and were resuspended in PBS with 5% (vol/vol) FBS at a density of 4 × 10<sup>6</sup> cells per ml for staining. Antibody to CD16/32 (2.4G2; produced ‘in-house’) was first added for 10 min on ice, before the addition of ‘cocktails’ of fluorophore-labeled or biotin-labeled monoclonal antibodies (monoclonal antibodies used for this are identified in Supplementary Table 1), followed by incubation for 45 min on ice. After samples were washed, biotin-labeled antibodies were detected by incubation for 15 min on ice with Qdot 655–streptavidin. For intracellular staining of Bcl-6, surfaces of cells (CD138<sup>−</sup>B220<sup>+</sup>CD38<sup>+</sup>GL7<sup>−</sup>) were individually sorted by flow cytometry into 96-well plates containing 5 µl RT-Pre-Amplification Master Mix (2.5 µl CellsDirect 2x Reaction Mix; 0.1 µl SuperScript III RT/Platinum Taq CellsDirect One-Step qRT-PCR kit; Invitrogen); 0.25 µl pooled 0.5 µM outside gene-expression primers and outside primers for analysis of the V<sub>J</sub>1.1 and V<sub>J</sub>1.186.2 BCR; 2.15 µl diethylpyrocarbonate-treated double-distilled water (primers and Taqman assays, Supplementary Table 2). As negative control for each plate, at least four wells per plate had no cell and were processed throughout the procedure. Therefore, a total of 384 individual negative data points were included for each plate. The reverse transcription was performed at 50 °C for 15 min, followed by 95 °C for 2 min, then 22 cycles of 95 °C for 15 s, 60 °C for 4 min. The preamplified cDNA was diluted five times in Tris-EDTA buffer and then was processed for analysis of gene expression and the BCR.

For multiplex gene-expression analysis, the BioMark Real-time PCR system (Fluidigm) was used with TaqMan Universal PCR Master Mix (Applied Biosystems) and an inventoried TaqMan gene-expression assay in 96.96 dynamic arrays (primers and Taqman assays, Supplementary Table 2). Single cells that did not express at least two of the reference genes (Gapdh, Actb and B2m) were removed from the analysis (<5% of cells). The cycling threshold (Ct) values from individual cells were transformed into relative mRNAB fluorescence abundance by subtraction of the Ct value from a baseline of 30, followed by conversion to a numerical value at 2<sup>−Ct</sup> for display on log scale.

For BCR analysis, 1 µl of the preamplification product was used for an amplification reaction in a volume of 10 µl containing 2 µM Taq polymerase, 200 µM of each dNTP (Roche), 1 mM Tris- HCl, 1.5 mM MgCl<sub>2</sub>, 25 mM KCl and 0.8 µM of the nested primers VH186-b (sense) (5′-CTCGGACACAGACTCACAG-3′) and Cgl-b (antisense) (5′-CACAGCGCCGGGAGAAATG-3′) for amplification of the gene encoding the heavy chain with the following PCR program: 95 °C for 5 min then 40 cycles of 95 °C for 15 s, 55 °C for 45 s, 72 °C for 90 s, ending with 72 °C for 5 min. PCR products were purified (ExoSAPT-IT; USB) and then were sequenced with a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) with 3.2 pmol of the second-round PCR antisense primer Cgl-b for heavy chain products. Sequence products were purified (BigDye XTerminator purification kit, Applied Biosystems) and run on a 3130 genetic analyzer (Applied Biosystems). Gene segments were assigned as encoding variable, diversity and joining regions through the use of the IgBLAST tool (National Center for Biotechnology Information) and Kabat nomenclature. Single-cell nucleotide sequences underwent near-neighbor alignment with the Clustal Omega multiple-sequence-alignment program and, on the basis of the nucleotide sequence in the BCR junctional regions, were assigned a clone and cell identification. Phylogenetic trees were generated with the Dendroscope 3 interactive viewer<sup>39</sup>.}

<sup>39</sup>t-SNE. For gating and visualization, gene-expression data files were compiled into flow cytometry standard files (with the CsvToFcs program for the conversion of comma separated value files into flow cytometry standard files; GenePattern genomic analysis platform of the Broad Institute) and were analyzed with FlowJo software. t-SNE<sup>40</sup> analysis of gene expression in GC B cells
(n = 372) (in the 30-Ct format) was done with the CYT tool (from the Péter laboratory of Columbia University) running with MATLAB software, with seven selected parameters (Aicda, Bcl6, Cd79b, Cd83, Cxcr4, Mki67 and Polh). t-SNE creates a two-dimensional representation of data points in which the closest neighbors in the high-dimensional space are plotted in close proximity, which conserves the local similarities between data points. The t-SNE coordinates of every data point were collated to the transformed gene-expression which conserves the local similarities between data points. The t-SNE closest neighbors in the high-dimensional space are plotted in close proximity, t-SNE creates a two-dimensional representation of data points in which the change in expression between consecutive stages was calculated as the absolute value of the change in the E.I. (as ‘fold’ values: \(2^{\Delta E \text{I}}\), presented as ‘\(\pm\)’ depending on the direction of change. Volcano plots were then created for every transition by plotting of the \(P\) value of the Mann-Whitney comparison versus the change in expression (‘fold’ values) for every gene. Genes that change in expression of >1.5-fold (gain or loss) and a \(P\) value of <0.05 for at least one of the transitions were selected, and the change of every transition (‘fold’ value) is presented as a heat map (Gene-E matrix visualization and analysis platform; Broad Institute).

Adoptive transfer. Single-cell suspensions pooled from the spleen and draining LNs of primed donor animals (C57BL/6 (B6) or B6.CD45.1) 10 weeks after immunization with NP-KLH were prepared. For transfer of non-specific class-switched memory B cells into B6.Rag1\(^{-/-}\) recipient mice, cell suspensions were stained with a ‘cocktail’ of monoclonal antibodies (Supplementary Table 1), and class-switched memory B cells (CD8\(^{-}\)Gr-1\(^{-}\)IgM\(^{-}\)IgD\(^{-}\)CD4\(^{+}\)CD19\(^{-}\)GL7\(^{-}\)) and CD4\(^{+}\)CD44\(^{hi}\) memory T cells (CD8\(^{-}\)Gr-1\(^{-}\)IgM\(^{-}\)IgD\(^{-}\)CD19\(^{-}\)CD4\(^{+}\)CD44\(^{hi}\)) were sorted separately into complete DMEM culture medium by flow cytometry. Spleen cell suspensions from congenically marked B6.MD4 mice were prepared at the same time, and each recipient B6.Rag1\(^{-/-}\) mouse was given intraperitoneal transfer of approximately 1 \(\times\) 10\(^{5}\) class-switched memory B cells, 7.5 \(\times\) 10\(^{4}\) CD4\(^{+}\)CD44\(^{hi}\) memory T cells and 25 \(\times\) 10\(^{6}\) B6.MD4 spleen cells. Recipient mice were immunized shortly after transfer with 100 \(\mu\)g NP-KLH in MPL adjuvant intraperitoneally (or were given adjuvant only). Donor B cell progeny were analyzed in the spleen of recipient mice 7 days after transfer. The various enrichment procedures had no significant effect on the outcome of these experiments.

Analysis of circle transcripts for C\(_{\mu}\)-Iy2b. IgG2b+ NP-specific B cells with different levels of expression of CD38 and GL7 from day 5 after priming or days 3 and 5 after boost (adjuvant) were isolated. Thirty cell samples were sorted for preamplification in a 5 \(\mu\)l master mix as described above with 50 nM external forward primers (I\(_{\gamma 2b}\) F1, 5'-GCTCCACATGTAAGTTG-3') and reverse primers (C\(_{\mu}\) R1, 5'-GCCAGGTTGCTAGCTACTCTG-3'). Pre-amplification products were diluted 1:5 in Tris-EDTA buffer, and 1\(\mu\)l was used for amplification of Gapdh and cDNA of circle transcripts for C\(_{\mu}\)-Iy2b by quantitative PCR. The reaction mix consisted of 5\(\mu\)l SYBR green reaction mix (PerfeCTa SYBR Green FastMix; Quanta Biosciences), 250 nM internal forward primers (I\(_{\gamma 2b}\) F3, 5'-CAGAGGGGCTTCTCAAGAACATA-3'), or Gapdh F, 5'-GGATGGAAGGGAGTGATTG-3') and reverse primers (C\(_{\mu}\) R3, 5'-AATGGTCCTGGCGAGGAGATT-3'), or Gapdh R, 5'-TGGCGCTAGTGAGCTCAGA-3'), or Gapdh R, 5'-TGGCGCTAGTGAGCTCAGA-3', and 3.5\(\mu\)l water. MicroAmp Fast 96-well plates (Applied Biosystems) were used in a StepOnePlus Real Time qPCR thermocycler (Life Technologies). The following program: 10 min at 95 °C, then 40 cycles of 30 s at 95 °C followed by 1 min at 60 °C, and the generation of a melting curve by an increase from 60 °C to 95 °C in increments of 0.3 °C. Gapdh amplicons were detected with a Ct value of approximately 25–30, and circle transcripts for C\(_{\mu}\)-Iy2b were detected with a Ct value of 13–16. Products were separated by electrophoresis through a 2% (wt/vol) agarose gel to verify they matched the expected amplicon sizes (approximately 90 base pairs for Gapdh and 300 base pairs for C\(_{\mu}\)-Iy2b). While Gapdh transcripts were detected in all samples sorted, circle transcripts for C\(_{\mu}\)-Iy2b had either maximal or negative expression; thus, positive samples included at least 1 of 30 sorted cells that had recently switched from IgM to IgG2b, but negative samples probably contained none.

Statistics. Mean values, s.e.m. values, unpaired t-tests and Mann-Whitney tests were calculated and graphed with Prism software (GraphPad). A \(P\) value of less than 0.05 was considered statistically significant.