A Temporal Switch in the Germinal Center Determines Differential Output of Memory B and Plasma Cells

Highlights

- Long-lived memory B cells are made predominantly in the early germinal center (GC)
- Long-lived bone-marrow-resident plasma cells are made very late in the GC response
- Subsets of memory B cells are also produced in a temporal order
- A substantial fraction of long-lived IgM memory B cells are made prior to GC onset

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In Brief

Generation of memory B and plasma cells is critical for protective immunity. Shlomchik and colleagues demonstrate that the B cell response shifts dramatically from generation of memory B cells at early time points to that of long-lived, bone-marrow-resident plasma cells at late time points.

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A Temporal Switch in the Germinal Center Determines Differential Output of Memory B and Plasma Cells

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SUMMARY

There is little insight into or agreement about the signals that control differentiation of memory B cells (MBCs) and long-lived plasma cells (LLPCs). By performing BrdU pulse-labeling studies, we found that MBC formation preceded the formation of LLPCs in an adoptive transfer immunization system, which allowed for a synchronized Ag-specific response with homogeneous Ag-receptor, yet at natural precursor frequencies. We confirmed these observations in wild-type (WT) mice and extended them with germinal center (GC) disruption experiments and variable region gene sequencing. We thus show that the GC response undergoes a temporal switch in its output as it matures, revealing that the reaction engenders both MBC subsets with different immune effector function and, ultimately, LLPCs at largely separate points in time. These data demonstrate the kinetics of the formation of the cells that provide stable humoral immunity and therefore have implications for autoimmunity, for vaccine development, and for understanding long-term pathogen resistance.

INTRODUCTION

Adaptive T-cell-dependent (TD) immune responses against foreign antigens (Ag) first generate plasmablasts (PBs), followed by a germinal center (GC) response that engenders both memory B cells (MBCs) and long-lived plasma cells (LLPCs) (De Silva and Klein, 2015; Nutt et al., 2015; Shlomchik and Weisel, 2012; Victoria and Nussenzweig, 2006; Taylor et al., 2012). Concurrently, some activated B cells undergo productive interaction with cognate T cells at the splenic T cell-B cell border or the interfollicular region of the lymph node, express the transcriptional repressor Bcl6, and migrate into the follicle to form a nascent GC (Kitano et al., 2011; Schwickert et al., 2011). GCs, initiating 3–4 days after immunization (Kerfoot et al., 2011; and see below), are sites where B cells undergo proliferation, V region gene somatic hypermutation, and class switch recombination (Jacob et al., 1991). In the GC, Ag-driven affinity maturation preferentially selects for the expansion of B cells with higher affinity for the cognate Ag (Le et al., 2008; Shih et al., 2002) possibly by competition for T cell signals (Gitlin et al., 2015; Liu et al., 2015), ultimately resulting in differentiation of mutated, higher-affinity MBCs and LLPCs.

Although it is widely accepted that bone marrow (BM) LLPCs are derived from GCs (Chan and Brink, 2012; Good-Jacobson and Shlomchik, 2010; Oracki et al., 2010; Takahashi et al., 1998), MBCs can also form in Bcl6−/− animals (Toyama et al., 2002), which lack GCs, or in response to T-cell-independent Ag (Obukhanych and Nussenzweig, 2006). Further, not all MBCs are of switched isotype (Dogant et al., 2009; Zuccarino-Catania et al., 2014) or display somatically mutated immunoglobulin (Ig) genes (Takahashi et al., 2001; Zuccarino-Catania et al., 2014; Tomayko et al., 2010). Although later-appearing isotype-switched MBCs are products of the GC, early-appearing cells with a MBC phenotype were posited to originate independently of GCs (Kaji et al., 2012; Pape et al., 2011; Takemori et al., 2014; Taylor et al., 2012). The contribution of each pathway to the total long-lived MBC compartment remains enigmatic.

It is unclear how differentiation of GC B cells (GCBCs) into MBCs and LLPCs is controlled (Allen et al., 2007a; De Silva and Klein, 2015; Nutt et al., 2015; Shlomchik and Weisel, 2012; Victora and Nussenzweig, 2012; Zotos and Tarlinton, 2012). Based largely on in vitro data, it has been proposed that CD40-mediated signaling and/or cytokine signals could control this decision, but there has not been agreement on whether such signals promote MBC versus LLPC formation. It was originally thought that differentiation could be controlled via affinity-based instructive B cell receptor (BCR) signals (Paus et al., 2006; Phan et al., 2005), but subsequently the same group elegantly showed that higher affinity increased overall
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proliferation—not antibody-forming cell (AFC) differentiation—of Ag-reactive cells, which in turn resulted in increased AFC numbers (Chan et al., 2009). Alternatively, differentiation might be a stochastic process, possibly metered by the number of cell divisions and/or signaling encounters (Hasboid et al., 2004). Given the many theories the resolution of this question remains a major unresolved question.

Some clues to the in vivo control of this process come from antibody (Ab) inhibition and genetic deletion studies. Blocking GCs with antibodies directed against CD40L or ICOSL results in a decrease of LLPCs (Takahashi et al., 1998) and deletion of CR1 and CR2 (Gatto et al., 2005), interleukin 21 receptor (IL-21R) (Linterman et al., 2010; Zotos et al., 2010), PD-1, PD-L1, and PD-L2 (Good-Jacobson et al., 2010), and CD80 (Good-Jacobson et al., 2012) allow GC initiation but affect proper GC maturation or progression. In all these cases, the loss of the late GC is correlated with diminished LLPC numbers, whereas MBC populations are mainly unaffected or even increased (reviewed in Good-Jacobson and Shlomchik, 2010). It could be that each of these signals differentially promotes LLPC versus MBC formation. Alternatively, it might be the case that these signals allow the GC reaction to reach a certain maturation point that favors LLPC generation.

To determine whether MBCs and LLPCs are generated at different time points during the response, here we used BrdU pulse labeling, an approach already successfully used to analyze the half-life of Ag-specific PCs (Manz et al., 1997) and the lifespan of MBCs (Schitteke and Rajewsky, 1990). We observed that long-lived immune progeny are generated in a sequential order: unswitched MBCs very early in the response, followed by switched MBCs and finally by a delayed appearance of isotype-switched BM LLPCs. We corroborated these findings using a combination of anti-CD40L Ab to destroy the GC at a key time point, as well as V region gene sequencing to match the content of early GCs with MBCs and late GCs with LLPCs. Based on these findings, we infer that less-committed humoral immune effector cells mainly derive from pre- or early GC reactions whereas cells of higher maturation stage are formed during late GC responses and propose a model that selection of proliferating GCBCs into the long-lived immune compartment is controlled by developmental stages within the GC reaction, resulting in a switch of output over time.

RESULTS

Kinetics of the Formation of Long-Lived Immune Effector Cells in a Synchronized Response

To determine when long-lived MBCs and LLPCs are stably formed, we induced a TD immune response in a transfer-immunization system (Figure 1A), which allowed for a synchronized response of a timed cohort of B cells with controlled BCR composition, and performed BrdU pulse labeling at different stages of the immune response. To this end, we transferred limiting numbers of 4-hydroxy-3-nitrophenyl acetyl (NP)-reactive naive B cells from B1-8i–/– BALB/cJ genetically targeted mice (henceforth referred to as B1-8 mice) into AM14 transgenic Vk8R genetically targeted BALB/cJ mice (henceforth referred to as transfer recipients), which have a fixed BCR that does not recognize NP-chicken gamma globulin (CGG) (Figure 1A). Upon immunization of recipient mice, WT-like numbers of ~12,500 splenic NP-reactive B cells (Figure S1; Le et al., 2008; Pape et al., 2011; Tomayko et al., 2010) participated in the response quickly, without continuous input of naive B cells into the primary response (Schwickert et al., 2009), thereby synchronizing the GC reaction.

Transfer recipients were immunized with NP-CGG and divided into 14 groups, each of which received three BrdU i.p. injections per day, with each group being injected for a different sequential 3-day period (Figure 1A); we refer to these periods as “labeling windows.” All mice were then analyzed at 8 weeks after immunization. This approach would label long-lived cells at the time point of their generation, because only cells that were in S-phase during the BrdU labeling window will have integrated BrdU into the nascent DNA within the first 1–2 hr after BrdU injection and only cells that stopped cycling during or immediately after the period of bioavailable BrdU would remain detectably BrdU labeled (Figure 1B) for at least 20 weeks (Anderson et al., 2006, and see below). 8 weeks after immunization, we enumerated splenic MBCs, MBC subsets, and BM LLPCs by multicolor flow cytometry (Figure S2); we posited that BrdU* MBCs or LLPCs identified in mice from a particular labeling window were stably formed during that time window of labeling (further discussed below). Thus, the percentage of BrdU* cells among either MBCs, MBC subsets, or LLPCs in a given window (Figures 1A, 1B, and S2) indicates the fraction of the total compartment that was formed during that window.
Overall, we found that Ag-specific cells that were labeled during the early part of the response and even beyond the peak of the GC were predominantly MBCs, with very few LLPCs that were destined to remain in the BM until at least week 8, whereas cells labeled during the late, post-peak of the GC response were overwhelmingly LLPCs. There was a pronounced pre-GC wave of IgM+ MBC formation (Figures 1C and S2A), with an initial peak during d0–d2 and 40% of IgM+ MBCs formed by d5. Peak formation of IgG1+ MBCs was between d6 and d8 after immunization, with ~22% of all MBCs generated in this 3-day window (Figures 1E and S2A) and about half of all IgG1+ MBCs were generated by d11, which was before the peak of the total GC response (d14, Figure 1F). Notably, ~9% of IgG1+ MBCs were formed by d5, indicative of a pre-GC origin (Figures 1E and 1F) and consistent with the early appearance of IgG1+ B cells with a memory-like phenotype very early after immunization (Figure 1E; Inamine et al., 2005). These data demonstrate that such IgG1+ MBCs that formed very early in the response persist for at least 8 weeks (Figure 1E).

To better understand whether MBCs that formed at different time points emanated from a GC or extra-GC source, we measured the fraction of NP+IgG1+ B cells labeled during each BrdU window by sacrificing transfer-immunized recipient mice 1 hr after the last BrdU injection instead of 8 weeks later. From d8 to d38, the vast majority of labeled cells had a GC phenotype (Figure 1D), implying that long-lived BrdU+ cells formed after d6 originated mainly from the GC. In contrast, from d0 to d2, there were no detectable labeled NP+IgG1+ cells of a GC phenotype (Figure 1D), indicating that resting cells formed during that time window and most likely a substantial fraction formed in the next time window were indeed GC-independent long-lived MBCs (Figures 1C–1F and further discussed below). The percentage of BrdU+NP+ GCBCs was not different among individual labeling windows, indicating equivalent labeling throughout the whole GC reaction (not depicted).

In contrast to the early formation of MBCs, only 7.5% of the IgG1+ BM LLPC compartment was formed by d14 (Figures 1G and S2B). Instead, the majority was generated much later—between week 2 and week 5 after immunization (Figure 1G). Consistent with these conclusions, the delayed development of LLPCs was reflected in the kinetics of the appearance of total AFC numbers in the BM (Figure 1H). Indeed, even though labeling windows extended to 6 weeks after immunization, we could account for only ~75% of the total LLPCs in summing up all of the labeled cells, indicative of substantial LLPC generation even 5 weeks after immunization (Figure 1G), whereas we could similarly account for ~95% of MBCs, indicating little if any further generation beyond week 6 (Figures 1C and 1E). Furthermore, labeling of ~95% of MBCs of both isotypes throughout the course shows that BrdU pulse labeling was highly efficient. Additionally, we did not find differences in the frequency of NP-specific B cells in BrdU- versus PBS-treated mice at week 8 (not depicted), which confirms that there was no toxic effect of BrdU.

**Heterogeneity among MBCs Is Reflected in the Kinetics of Their Generation**

We previously showed that the NP-specific MBC compartment is comprised of at least three phenotypically and functionally distinct subsets, which can be distinguished by expression of CD80 and PD-L2 (Tomayko et al., 2010; Zuccarino-Catania et al., 2014). To assess whether there are distinct kinetics of formation of these distinct MBC subsets, we included staining for these two markers in our Ag-specific BrdU analysis of MBCs. Up to d5, mainly CD80+PD-L2+ MBCs were produced. Between d6 and d11, the most abundantly produced MBC subset was of CD80+PD-L2+ phenotype, whereas among the MBCs formed from d12 onward, most were CD80+PD-L2+ (Figures 1I and S2C). These data imply that qualitatively distinct effector functions (Zuccarino-Catania et al., 2014) of MBCs are reflected in the kinetics of their generation. This result is in line with findings from Kaji et al. (2012), who reported that the secondary immune response of adoptively transferred d40 MBCs led to 5–6 times more AFCs in recipient mice compared to adoptively transferred d7 MBCs.

CD73 is expressed on a subset of MBCs (Figure 1J; Tomayko et al., 2010), and it was suggested that CD73 expression marks GC-derived MBCs (Taylor et al., 2012). BrdU pulse labeling revealed, however, that among the IgG1+ MBCs formed between d0–d2 and d3–d5, about 60% were CD73+ (Figure 1K), indicating that MBCs formed prior to the GC reaction often express CD73. Similarly, among CD73+ MBCs, approximately 37% of IgM+ cells and 6% of IgG+ cells formed by d5, a time point prior to substantial GC formation (Figures 1F and 1L). Thus, CD73 expression is neither exclusive to (Figure 1K) nor specific for (Figure 1L) GC-derived MBCs, even though somatically mutated MBCs are enriched in CD73+ subset (Kaji et al., 2012).

**Validation of Experimental Design**

The adoptive transfer system provided sufficient numbers of cells to enable us to validate the assumptions underlying the short-term BrdU “window-labeling” approach (Figure 1A) we used to address long-term precursor-product relationships (Figure 1B). To validate the assumption that BrdU labeling of MBCs would become undetectable after further division, we injected transfer recipient mice from d6–d8 after immunization with BrdU 3 times daily, and 6 weeks later harvested and labeled splenocytes with VPD450. We cultured these cells with ODN1826 to stimulate division, then used VPD dilution to correlate BrdU staining and cell division by flow cytometry (Figure 2A). Most (~65%) cells had lost BrdU label by the first division and by the third division 92% of previously labeled cells had lost detectable BrdU (Figure 2B). Because GCBCs divide 3–4 times per day, even if it took 3 divisions to lose labeling, this still gave a resolution of a single day or less for the labeling windows, which is as postulated in the experimental design (Figures 1A and 1B). We further confirmed BrdU and 5-ethyl-2′-deoxyuridine (EdU) in vivo double labeling experiments to measure how many divisions a GCBC underwent in vivo from its last division in which it was BrdU labeled as a GCBC until achieving a resting MBC state. To evaluate this, we injected transfer recipient mice with BrdU for 1 day and then 1–4 days later with a single dose of EdU and harvested spleens 30 min later (Figure 2C). We found that by d1 only 4% and by d2 only 1% of BrdU+ cells with MBC phenotype were labeled with EdU (Figures 2D and 2E). Taking into account that ~1/4–1/3 of cycling GCBCs are in S phase at a given time (Allen et al., 2007b), this indicates that ~90% of labeled cells exited the cell cycle within 1 day and >95% within 2 days (Figure 2E). Considering that as many
T cell-B cell border, fewer Ig thus Ag-specific) B cells were proliferating inside the T cell zones 30 min after the last EdU injection and at each time point we assessed the micro-anatomic location where active proliferation of Ag-specific B cells takes place at early time points. We in-

Micro-anatomic Locations of the Formation of Early MBCs

Given that MBCs are formed very early in the immune response (Figures 1C and 1E) and that these undoubtedly stem from proliferating precursors responding to Ag stimulation (Figure S2A), we assessed the micro-anatomic location where active proliferation of Ag-specific B cells takes place at early time points. We injected transfer recipients at d2–d6 after immunization three times with EdU to label proliferating cells. Mice were sacrificed 30 min after the last EdU injection and at each time point we found that most of the lambda light chain (Igλ) positive (and thus Ag-specific) B cells were proliferating inside the T cell zones and at the T cell-B cell barrier. Compared to T cell zones and the T cell-B cell border, fewer Igλ+ B cells were proliferating within B cell follicles at d2 of the response (Figures 3A and 3C). Because ~20% of the IgM+ MBCs are formed by d2 (Figure 1C), and the majority of proliferating Ag-specific B cells—containing presumptive MBC precursors—are located in the T cell zones, it is very likely that most early MBCs were generated inside T cell areas. Proliferation of Ag-specific cells within T cell zones was found throughout all analyzed time points, suggesting potential continued generation of extra-GC MBCs (Figure 3C) up to at least d6. On d3, small GCs were detectable, indicating that from this point on the GC could contribute to long-lived compartments, although that the majority of proliferating cells at d3 was outside the GC. From d4 onward, the majority of proliferating Igλ+ B cells was found within GCs (Figures 3B, 3C, and S3; Le et al., 2008).

MBCs and LLPCs Are Formed with Different Kinetics in WT Mice

Using the insights gained from validating and characterizing the cell-transfer BrdU labeling system, we sought to track the kinetics of B cell differentiation into MBCs and LLPCs in a polyclonal response. There are, however, limitations to this experi-

Disruption of Peak GC Reactions Diminishes LLPCs but Not MBCs

If our interpretation of the labeling data is correct, then if we were to abrogate the GC reaction after most of the MBCs were predicted to be formed, we expect this would have a differential and disproportional effect on LLPCs versus MBCs. To experimentally test this, we used anti-CD40L Ab to disrupt the peak GC reaction in the transfer-immunization system at d12–d14 (Figure 1F), by which time BrdU data indicate that most MBCs should have formed but most LLPCs would have yet to be formed (Figures 1C, 1E, and 1G). As predicted by the BrdU data, disruption of the GC at d12–d14 led to a significant reduction in numbers of NP-specific IgG1+ BM AFCs at 8 weeks after immunization (Figure 5A), with a marked—almost 90%—loss of higher-affinity AFCs (Figure 5B). In contrast, but also as predicted by the labeling data, total numbers of residual NP-specific B cells, which represent the MBC compartment, were not significantly affected (Figure 5C). Hence, as determined independently by experimental abrogation of the immune response,
most MBCs are formed by d12–d14 whereas most LLPCs had yet to form. In concert with our finding that CD80+/PDL2+ MBCs are mainly generated late in the immune response (Figure 1f), we found these cells to be the only subset whose frequency was reduced by anti-CD40L Ab treatment (Figure 5f). In contrast, the frequencies of CD80−/PDL2− (Figure 5d) and CD80+/PDL2+/ (Figure 5e) subsets were slightly but significantly elevated, which was an expected result because the overall number of MBCs (Figure 5c) remained unaltered.

Mutational Content Analysis Correlates GCBCs with Their Long-Lived Progeny over Time

Mutations accumulate in the V gene regions of GCBCs as a function of time or cell divisions (Takahashi et al., 2001), and the extent of mutations can be used as a footprint to compare populations of B cells that have undergone mutation. In particular, non-mutating resting populations including both MBCS and LLPCs should have numbers of mutations similar to those in the GCBCs that were their direct precursors at the time that those GCBCs differentiated. We used this as a third approach to connect GC time windows to the types of long-lived B cell products that were created by them. To this end, we compared the V region mutational content of Ag-specific IgG1+ GC (and non-GC) cells in B1-8 mice at d6–d8 and d18–d20 after NP-CGG immunization to the mutational content of IgG1+ MBCs and LLPCs formed at those time periods. We marked the latter cells during their formation in analogous fashion to the experiments in Figures 1 and 4, except we used EdU because this was compatible with sequencing of the labeled sorted cells (Figure 6A; Gittlin et al., 2015).

We sorted NP-specific IgG1+ GC and non-GC cells directly at d7 (the midpoint of the d6–d8 labeling window) and d19 after immunization and sorted NP IgG1+EdU+ splenic MBCs and BM LLPCs at week 8 from mice that had been given EdU at either d6–d8 or d18–d20 (Figure 6A). The frequencies of resulting EdU+ MBCs and BM LLPCs were comparable to frequencies obtained with BrdU labeling, indicating similar labeling efficiencies of EdU and BrdU. We then cloned and sequenced V.1 rearrangements from all of these sort-purified cell populations. The V.1 mutation frequency of EdU+ MBCs labeled during d6–d8 matched that of d6–d8 GCBCs, but neither that of non-GC B cells isolated at d7 nor of GCBCs harvested at d18–d20 after immunization (Figure 6B).

Conversely, the mutation frequency of EdU+ LLPCs formed during d18–d20 matched only that of d19 GCBCs. Furthermore, the mutational content of d7 GCBCs and MBCs derived at this time was indistinguishable from that of total MBCs at week 8, most likely because the majority of MBCs were made near this time window (Figures 1 and 4). These data thus independently support an earlier origin for most MBCs compared to the majority of LLPCs.

Gene Expression Analysis of Early and Late GCBCs

To gain insight in the mechanisms underlying this temporal switch in output of GC reactions, we compared RNA expression profiles of d8 and d18 GCBCs sorted from adoptive transfer recipients. These data revealed consistent differences with a fold change of 1.7 or greater in ~100 genes, with q value of <0.05 (Figure 7).

Notably, a number of these are annotated as signaling molecules and/or receptors that are known to be important in immune responses. In addition, there were several transcription factors and chromatin modifiers that were differentially expressed in early versus late GCs. Although extensive testing will be needed to determine the functions of these molecules in determining the different outputs of early versus late GCs, these data establish clearly that the two types of GCs are not just functionally but are indeed also transcriptionally distinct (Figure 7 and below).

DISCUSSION

Our results provide insight into the longstanding question of how the GC generates both MBCs and LLPCs, revealing that there is a time-dependent developmental switch in the output of the GCs, such that it first dedicates itself to MBC generation and later—long after its peak in size—switches to mainly producing LLPCs. These conclusions were reached by a combination of three strategies: pulse chase in vivo BrdU labeling, V region sequencing, and selective elimination of the GC response at d14 that resulted in marked reduction of LLPCs but not MBCs.

It had not been evident that the “mature” GC changes functionally in such a profound way over time. Previous studies might have missed this conclusion because their underlying hypotheses posited that there would be discrete instructional signaling that would govern the concurrent production of MBCs and LLPCs at the local cell level. Nonetheless, consistent with our results, various mutations in GC-related molecules result in a...
significant loss of LLPCs but not MBCs (reviewed in Good-Jacobson and Shlomchik, 2010), including CR1 and CR2 (Gatto et al., 2005), IL-21R (Linterman et al., 2010; Zotos et al., 2010), PD-1, PD-L1, and PD-L2 (Good-Jacobson et al., 2010), and CD80 (Good-Jacobson et al., 2012). Because these mutants also all share the phenotype of premature termination of the GC response, we favor the notion that multiple signals are required to allow the GC to mature to a certain point at which the milieu becomes favorable for LLPC generation (Shlomchik and Weisel, 2012). Notably, CD40 signals, Ag affinity, and certain cytokine signals have all been investigated, without clear results to implicate them directly in an instructive program for either MBC or LLPC differentiation (Chan and Brink, 2012). Of note with respect to other controversies surrounding GC function, because formation of MBCs and LLPCs is greatly separated in time, it seems unlikely that MBCs and LLPCs are created alternatively via asymmetric division, as has been suggested to occur (Barnett et al., 2012).

Figure 3. Immunohistological Analysis of Early MBC Formation
(A and B) Transfer recipients were injected three times with EdU at d2 (A) and d6 (B) after NP-CGG immunization to label proliferating cells. Spleens were harvested 30 min later and sections were stained for B cells (B220, green), T cells (CD4, light blue), proliferation (EdU uptake, red nuclei), and NP specificity (Igλ, dark blue). Areas of active proliferation of Ag-specific B cells are marked with color-coded rectangles in the overview of representative areas (left) and are magnified (right) without depicting B220 and CD4 staining.
(C) Quantification of micro-anatomic location of proliferating Igλ⁺ B cells from three individual whole reconstructed splenic sections of two mice per time point (Figure S3A). Error bars represent ± SEM. GC phenotype was confirmed by PNA positivity on consecutive sections (Figure S3B). No significant expansion of EdU⁺Igλ⁺ B cells was observed in mice not given EdU injection, NP-CGG immunization, or without B1-8 B cell transfer (Figure S3C). Scale bars represent 200 μm.
Our results modify our view of how the GC evolves and in this way raise a new set of questions. How do changes in the GC milieu support this time-dependent shift in the generation of long-lived B cell progeny? Evolution in the tendency of the GC to produce MBCs toward LLPCs could be B cell intrinsic, with cells counting divisions in some way and this in turn influencing in a stochastic fashion the likelihood to differentiate into a MBC versus a LLPC (Hasbold et al., 2004). This is in accord with concepts elaborated by Hodgkin and colleagues based on in vitro data, including recent elegant single-cell studies of short-term B cell cultures (Duffy et al., 2012). In this regard, mRNAs encoding several key transcription factors and co-factors related to GC, MBC, and LLPC identity are differentially expressed in early and late GCBCs, including Irf8, Egr1, Meif2b, Sh3bgrl2 (whose protein is an interacting partner with Irf8 [Yoon et al., 2014]), and Zbtb20 (also known as Zfp288), which has been recently implicated in the development of LLPCs (Chevrier and Corcoran, 2014; Nutt et al., 2015; Wang and Bhattacharya, 2014).

Previously, BCR signaling in the GC was shown to be highly attenuated, with signaling observed only in the G2-M phase (Khalil et al., 2012), but this has so far been studied only at the peak of the GC reaction. Our transcriptional analysis shows that a number of key molecules affecting signaling are differentially regulated in early and late GCBCs, including Irf8, Egr1, Meif2b, Sh3bgrl2 and also Lck and Sh2d2a (which encodes a T-cell-specific adaptor protein)—both thought to be important for T cell receptor signaling (Granum et al., 2008) but which are evidently differentially regulated in GCBCs. Further studies are needed to address outcomes of BCR-mediated Ag-binding in late GCBCs.

There could also be temporal evolution of signals delivered by other cells to B cells in the GC. Notably, the total number of T cells per GC as well as GC T cell density declines significantly at d16 after immunization (Wollenberg et al., 2011), a time when the GC reaction is switching its output. In addition, T follicular regulatory cells accumulate with time in the GC (Ramiscal and Vinuesa, 2013; Wollenberg et al., 2011). Therefore, T-cell-derived signals might change with GC maturation due to time-dependent changes in quantity or quality. Commensurate with this is the downregulation of Stat4 and Il10ra in late GCBCs, which could affect responsiveness to key Tfh cytokines such as IL-21 and IL-10 (De Silva and Klein, 2015; Linterman et al., 2010; Ramisical and Vinuesa, 2013).

Regardless of the source of evolving external or B-cell-intrinsic signals that might shift in time, a common denominator in the B cell could be NF-κB signaling, because it was recently shown that specific subunits are required in B cells at different stages during the GC response (Heise et al., 2014). Another factor could be lymphocyte metabolic pathways, which can differ as a function of differentiation and activation (Caro-Maldonado et al., 2012). The GC reaction is dynamic in size and cell composition with the majority of GCs getting smaller at d14 (Rao et al., 2002). It is therefore conceivable that energy supply and oxygen availability might vary as a function of time, perhaps providing cues for selective differentiation. Notably, of the ~100 differentially regulated genes, 20 of them are annotated as affecting metabolism (Figure 7). All of the potential modifiers mentioned above are mutually non-exclusive and any or several of them could facilitate the general shift of the GC output over time. The current findings now help to refocus the research questions on evaluating these possibilities, which should ultimately lead to a better understanding of the origins of both MBCs and LLPCs and the differentiation process in the GC.

Several groups have noted that MBC-phenotype cells can be observed very early in the immune response. MBCs can form in mice lacking Bcl6 and therefore GCs (Kaji et al., 2012; Toyama et al., 2002), although it is possible this wouldn’t occur if Bcl6 were present. Further supporting the notion, putatively GC-independent IgG1+ cells with a surface phenotype similar to that of MBCs were detected by d10 (Iinami et al., 2005).
and IgM⁺ B cells with memory phenotype were found by d5 (Pape et al., 2011). It is now apparent that GCs can form as early as d3–d4, though not earlier than this (Kerfoot et al., 2011, and see above). Our findings demonstrating formation via stable BrdU labeling before the GC reaction has started definitively support the existence of GC-independent MBC formation in normal, $Bcl6$-intact animals; EdU labeling studies suggest that most form in the T cell zones and at the T cell-B cell border. In addition, our data shed light on the fate and utility of these cells. We show that these cells join the long-lived MBC compartment: a substantial fraction of IgM⁺ MBCs (and some IgG1⁺ MBCs) are made during d0–d2 and remain at least for 8 weeks. The surface phenotype of these MBCs generated very early in the response is also of interest. The great majority of these early MBCs lack expression of CD80 and PD-L2, yet most of them express CD73. These results, coupled with our recent observations on the functions of MBC subsets (Zuccarino-Catania et al., 2014), indicate that these early, mainly unmutated and unswitched MBCs are specialized to efficiently adapt to antigenic variants upon re-exposure, as they can undergo secondary GC reactions (Zuccarino-Catania et al., 2014). Furthermore, the expression of CD73 on these pre-GC-derived MBCs clearly shows that CD73 is not a suitable marker to identify GC-derived MBCs, in contrast to what was previously thought (Taylor et al., 2012). The evident design of the system, with generation of early MBCs followed by late LLCPC generation, presents a seemingly rational adaptation. Early generation of MBCs both within and outside of the GC could be a successful strategy for reinforcing the response to pathogens that persist beyond the earliest adaptive immune responses. Such quickly formed MBCs could presumably rejoin the response should the pathogen persist, though this has yet to be directly demonstrated. Early differentiation of long-lived MBCs, with the resultant paucity of mutations in V gene regions, probably prevents the humoral memory compartment in total from becoming overly committed to the initial immunogen, potentially a more optimal strategy for later responses to genetically variant pathogens.

Conversely, generation of LLCPCs occurs at a time when a prolonged selective process in the GC has allowed for a fine-tuning and commitment to the best possible affinity for the original Ag. This design is appropriate for cells that are terminally differentiated. The delay in making effective LLCPCs might also explain why optimally protective long-term Ab responses to certain vaccines (e.g., hepatitis B) take so long to appear. It is important to point out that our results examine the BM PC compartment only at week 8 and do not bear on AFCs that might have been present at earlier time points. It has been postulated that BM-resident AFCs are generated early in the response, followed by selective loss of low-affinity variants from d14 to d28 whereas higher-affinity cells persisted from d14 to d35 (Smith et al., 1997). Because we found that 7%–9% of LLCPCs resident in the BM at week 8 were generated by d14, our data are not in conflict with the concept of early

Figure 5. Disruption of Peak GC Reaction Diminishes LLCPCs but Not MBCs

(A–C) Transfer recipients were injected i.p. with 350 μg anti-CD40L Ab or hamster control IgG at d12, d13, and d14 after NP-CGG immunization. Each symbol represents one mouse and lines are means. Numbers of low-affinity (NP₁₆-BSA) (A) and high-affinity (NP₂-BSA) (B) BM AFCs per 10⁶ BM cells were measured by ELISpot and numbers of live NP⁺CD38⁺CD95⁺CD19⁺ splenic B cells were quantified by flow cytometric analysis (C) 8 weeks later.

(D–F) Frequency distributions of MBC subsets, as distinguished by their expression of CD80 and PD-L2, were analyzed by flow cytometry. Shown are representative results of one out of two independent experiments.
BM AFC generation (Smith et al., 1997) and it seems likely that long-lived, higher-affinity LLPCs that are generated later in the response replace or outnumber the initial lower-affinity cohorts.

Finally, our results might also be informative for designing vaccine strategies. Some vaccines demonstrate only short-lived Ab responses and protection. If the goal is to generate Ab-mediated long-lived protection, then adjuvant and Ag designs—as well as...
immunization schedules—should be adjusted to prolong late GC responses with resultant production of more and better LLPCs. In this regard, Kasturi et al. (2011) have demonstrated that immunization with immobilized Ag on synthetic nanoparticles together with TLR7 and TLR4 ligands induces a synergistic increase in numbers of LLPCs and long-lived Ab titers, which correlates with enhanced and sustained late GC responses. Our studies probably elucidate the underlying connection between these two observations.

The converse might be true in the case of influenza, because mutating MBCs contribute to early PB responses to antigenically drifted viruses (Wrammert et al., 2008), whereas Ab produced by LLPCs will be of little long-term value as quickly as the next flu season. In this situation, vaccines that promoted enlarged early GC responses, coupled with approaches to forestall GC maturation, would be more optimal, because this should yield relatively more MBCs. As appreciated also by others (De Silva and Klein, 2015), further elucidation of molecular changes that could modulate GC quality, kinetics, and subsequent output could empower us to manipulate the GC reaction and impact efforts to prevent infectious diseases.

**EXPERIMENTAL PROCEDURES**

**Mice, Immunization, and In Vivo Treatment**  
Use of B1–8R−/− genetically targeted BALB/cJ mice as B cell donors and AM14 Transgenic (Tg) × Vκ8R genetically targeted BALB/cJ mice as recipient strain was recently described (Zuccarino-Catania et al., 2014). All mice were maintained under specific-pathogen-free conditions and all animal experiments were approved by the Yale or University of Pittsburgh Institutional Animal Care and Use Committee. 6- to 12-week-old mice were i.p. immunized with 50 μg of NP-CGG precipitated in alum. To disrupt GC reactions, mice were i.p. injected once a day at d12–d14 with 350 μg purified anti-CD40L Ab (MR1) in PBS or Armenian hamster IgG (Innate) as control. To prevent GCs from re-appearing, mice were continuously i.p. injected with 125 μg Ab once a week until the end of the experiment. 5-bromo-2'-deoxyuridine (BrdU, Sigma) and 5-ethyl-2'-deoxyuridine (EdU, Invitrogen) were dissolved in sterile PBS and 0.75 mg to 1 mg in a volume of 200 μl were injected i.p.

**Adoptive B Cell Transfer**  
Splenocytes from B1–8R−/− genetically targeted BALB/cJ mice were depleted of T cells by incubation with supernatants of rat IgM anti-mouse CD4 and rat IgM anti-mouse CD8 Ab-producing cell lines and the addition of Low-Tox rabbit complement (Cedarlane) followed by Percoll (GE Healthcare) gradient purification. Flow cytometric analysis was performed to determine the frequency of NP-binding B cells. An equivalent of 2 × 10^5 untouched NP-reactive B cells was then transferred into tail veins of AM14 Tg × Vκ8R genetically targeted BALB/cJ mice.

**Flow Cytometric Analysis, Cell Sorting, and ELISpot Assay**  
Flow cytometric analysis and ELISpot assays were performed essentially as described (Good-Jacobson et al., 2010; Zuccarino-Catania et al., 2014). EdU detection was performed with the Click-it EdU Alexa Fluor 555 Imaging Kit (Invitrogen). 40× tiled images of whole splenic sections were acquired with an IX83 fluorescent microscope (Olympus) and image analysis was performed with cellSens Dimension software (Olympus) with the count and measure module.

**In Vitro B Cell Stimulation**  
B cell purification was performed with the EasySep Mouse B Cell Enrichment Kit (StemCell Technologies). 1 × 10^6 cells/ml were incubated in PBS with 0.5% (v/v) fetal calf serum (FCS, HyClone) and 4 μM of BDHorizon Violet Cell Proliferation Dye 450 (VPD450, BD Biosciences) for 5 min at 37°C and the reaction was quenched with 3 volumes of FCS for 1 min at 37°C and cells were washed twice in RPMI 1640 medium. Cells were cultured in the presence of 5 μg/ml CpG ODN 1826 (5′-tcccagcagcttccagct-3′; Invivogen) for 3–4 days at 37°C and then subjected to flow cytometric staining.

**Sequencing and Immunohistological Analysis**  
V1.1 sequencing was performed essentially as described (Anderson et al., 2007). Immunohistological analysis was performed essentially as described (Kerfoot et al., 2011). EdU was detected with the Click-it EdU Alexa Fluor 555 Imaging Kit (Invitrogen). 40× tiled images of whole splenic sections were acquired with an IX83 fluorescent microscope (Olympus) and image analysis was performed with cellSens Dimension software (Olympus) with the count and measure module.

**Statistics**  
The Mann-Whitney nonparametric, two-tailed test was used for statistical analyses. Significance was determined at the 95% confidence level and is defined as follows: **p < 0.001; **p = 0.001–0.01; *p ≥ 0.01–0.05; n.s., p > 0.05. Interpolation of missing data (Figure 4B) was performed with the spline function in the R statistical package (http://www.R-project.org).

**ACCESSION NUMBERS**  
The microarray data have been deposited in NCBI GEO under accession number GEO: GSE78502.

**SUPPLEMENTAL INFORMATION**  
Supplemental Information includes four figures and Supplemental Experimental Procedures (including Ag, Ab, and detection reagents) and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.12.004.

**AUTHOR CONTRIBUTIONS**  
F.J.W. and M.J.S. designed research; G.V.Z.-C. performed experiments and gave conceptual advice; M.C. performed bioinformatical analysis; and F.J.W. implemented and analyzed all experiments and wrote the manuscript together with M.J.S.

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**Figure 7. Gene Expression Profiling of Early and Late GCBCs**  
On d8 and d18 after NP-CGG immunization of transfer recipients, 3 × 10^5 live CD19+NP kappa light chain + CD38−CD95− splenic GCBCs were sorted per sample and subjected to gene expression profiling using illumina mouseWG-6 v2.0 expression beadchip arrays. Heatmap of differentially expressed genes of early (d8) and late (d18) NP reactive late GCBCs. Each column represents an independent replicate. Genes with a statistically significant (false-discovery rate q < 0.05) change in expression of ≥ 1.7 are displayed and were grouped by function.

REFERENCES


A Temporal Switch in the Germinal Center Determines Differential Output of Memory B and Plasma Cells

Florian J. Weisel, Griselda V. Zuccarino-Catania, Maria Chikina, and Mark J. Shlomchik
**Figure S1, related to Fig. 1 and 2. Adoptive transfer system to adjust NP reactive precursor frequency to WT levels.** AM14 transgenic x Vk8R genetically targeted BALB/cJ mice harbor an irrelevant monoclonal population of B cells and therefore are unable to mount endogenous NP-specific immune responses but otherwise display normal lymphoid architecture (Prak and Weigert, 1995; Shlomoichik et al., 1993) with reduced numbers of B cells. These mice were adoptively transferred with an equivalent of 2x10^6 NP reactive B cells from B1-8^i/- genically targeted BALB/cJ mice, carrying a germline encoded, unmaturated Vh186.2 site-directed transgene that encodes a BCR with moderate affinity for the hapten NP when paired with λ1 light chains (Sonoda et al., 1997), to allow donor B cell derived NP-specific immune responses. (A) 24h after adoptive transfer recipients were sacrificed and splenocytes were stained to determine the frequency of Igλ^+ NP-binding B cells. FACS plots are gated on live CD19^+ cells and each plot represents an individual AM14 transgenic x Vk8R genetically targeted BALB/cJ mouse either transferred with NP-reactive B cells (top row; B cell tx) or injected with transfer buffer only (middle row; No tx). Naive BALB/cJ WT mice served as control (bottom row). Numbers adjacent to outlined areas indicate percent gaged population. (B) Quantification of frequencies of NP-reactive B cell displayed in (A) indicate a WT-like precursor frequency of on average of about 12.5x10^3 NP-specific B cells per recipient spleen at the time point of immunization.
Figure S2, related to Fig. 1. Gating strategy for the detection of BrdU* MBC, MBC subsets and LLPC, 8 weeks after immunization of transfer recipients. Transfer recipients were immunized with NP-CGG in Alum (left column) or Alum only (middle column) 24 h after NP-reactive B cell transfer and i.p. injected with BrdU or PBS at indicated time points as outlined in Fig. 1A. Mice immunized with NP-CGG in Alum without NP-specific B cell transfer (right column in A and B; no tx) served as controls. Single-cell suspensions of red blood cell-depleted splenocytes (A, C) or bone-marrow cells (B) were analyzed 8 weeks after immunization by flow cytometry to determine the frequency of BrdU* cells. (A) MBC were defined as EMA+ NP+ B220+ CD38+ CD95+ and IgG1* or IgM* cells. (B) BM LLPC were stringently defined as EMA+ B220+ CD138+ NPsurf- NPintracellular- IgG1* cells. The distribution of CD80 and PD-L2 of EMA+, NP+ CD19+ BrdU* cells was assessed in (C). Arrows indicate subsequent gating of populations and numbers next to outlined areas indicate percent gated population. Fluorescent minus one (FMO) controls were included to prove specificity of the Ab intentionally excluded from the staining solution. Not applicable (NA) is depicted instead of subsequent gating if parental gate contains virtually no cells.
Figure S3, related to Fig. 3. Validation and quantification of histological analysis. Transfer recipients were injected with 0.85mg EdU at 6.5, 3.5 and 0.5h prior to harvesting spleens at indicated days post NP-CGG immunization. (A) Representative example of region definition and measurement. Regions of interest (ROI) were drawn around white pulps and the area of B cell zones was determined and measured by staining intensity using the count and measure analysis module of cellSens Dimensions software (Olympus). Areas of T cell zones where measured in analogous fashion (not depicted). Proliferating Ag specific (Igλ⁺ EdU⁺) B cells were counted within each area. (B) Clustered proliferation of Ag specific B cells within B cell follicles (color-coded, dotted regions) was defined as GC reaction if counterstaining for peanut agglutinin (PNA) was positive on consecutive sections. The markers B220, EdU and Igλ (not depicted in right panel) were used to identify these regions on the consecutive sections (color-coded, dotted regions). Examples for d4 (upper panel) and d6 (lower panel) are shown. (C) Validation of specificity of measured response. Transfer recipients not given EdU injection (left panel) or without NP-CGG immunization (middle panel) or without B1-8 B cell transfer (right panel) did not show significant expansion of Igλ⁺ B cells. These controls indicate that the observed expansion of Igλ⁺ B cells was due to Ag-mediated activation of adoptively transferred B1-8 B cells. Scale bars are 200µm.
Figure S4, related to Fig. 4. Gating strategy for the detection of BrdU+ MBC and LLPC, 8 weeks after immunization of WT mice. BALB/cJ WT mice were immunized with NP-CGG in Alum (left column) or Alum only (right column) and i.p. injected with BrdU or PBS at indicated time points as depicted in Fig. 1A. Single-cell suspensions of red blood cell-depleted splenocytes (A) or bone marrow cells (B) were analyzed 8 weeks after immunization by flow cytometry to determine the frequency of BrdU+ cells. (A) MBC were defined as EMA: NP+ B220+ CD38+ CD95- and IgG1+ or IgM+ cells. (B) BM LLPC were stringently defined as EMA: B220- CD138+ NPsurface- NPintracellular IgG1+ cells. Arrows indicate subsequent gating of populations and numbers next to outlined areas indicate percent gated population. Fluorescent minus one (FMO) controls were included to prove specificity of the Ab intentionally excluded from the staining solution. Not applicable (NA) is depicted instead of subsequent gating if parental gate contains virtually no cells.
**Antigens, antibodies and detection reagents**

Chicken $\gamma$ globulin (CGG; Sigma-Aldrich) or Bovine serum albumin (BSA) was haptenated with nitrophenyl (NP)-hydroxysuccinimide ester (Cambridge Research Biochemicals). Allophycocyanin, Phycoerythrin and BSA were haptenated with nitro-iodo-phenyl (NIP) – hydroxysuccinimide ester. The haptenation ratios of NP or NIP to proteins were determined by spectrometry. NP-33-CGG was used for immunizations. The following reagents were prepared and/or conjugated in our laboratory: NIP$_5$-BSA-Alexa Fluor 680; anti-IgM (B7-6) Alexa Fluor 680 and Pacific Blue Ab; anti-CD19 (1D3.2) Pacific Blue or Alexa Fluor 647 Ab; anti-CD80 (16-10A1) Alexa Fluor 488 Ab; Anti-B220 (RA3-6B2) Alexa Fluor 488 or Alexa Fluor 647; anti-kappa (187.1) Pacific Blue; Alexa Fluor 647 or unconjugated anti-CD16/CD32 (2.4G2) Ab. Unconjugated polyclonal Goat anti-lambda Ab was purchased from Southern Biotech and conjugated to Alexa Fluor 680 and Pacific Blue in our laboratory. Unconjugated peanut agglutinin (PNA) was purchased from Vector Laboratories and conjugated to Alexa Fluor 488 in our laboratory. Anti-PDL2 (TY-25) biotin Ab, anti-CD38 (90) PE or biotin Ab were purchased from Biolegend. Anti-IgG1 (A85-1) FITC or V450 Ab and anti-CD138 (281-2) biotin or PE Ab were ordered from BD Biosciences. Anti-B220 (RA3-6B2) APC-Cy7 Ab, anti-CD19 (ID3) APC-Cy7 Ab, anti-CD95 (Jo2) Pe-Cy7 Ab and anti-CD73 (TY/23) PE Ab were from BD Pharmingen. Anti-BrdU (MoBU1) Alexa Fluor 647 Ab was from Invitrogen. Polyclonal anti-IgG1-AP Ab was purchased from Southern biotech. Ab-producing cell lines 174.2 (rat IgM anti-mouse CD4) and 31-68.1 (rat IgM anti- mouse CD8) were grown in RPMI 1640 media to generate antibody containing supernatants (Ceredig et al., 1985) for complement mediated cell depletion.
**BrdU detection by multicolor flow cytometry**

1x10^7 red blood cell depleted splenocytes were incubated with anti-CD16/CD32 Abs and EMA in staining buffer (SB; 1xPBS, 3%FCS, 2mM EDTA, 0.02% NaN₃) for 15min on ice followed by 10min exposure to fluorescent light. Cells were washed and stained for surface antigens for 30min on ice in SB and then washed in SB. Cell pellets were resuspended in 500µl cold 0.15M NaCl and 1.2ml of pre-cooled 100% ethanol were dropwise added while vortexing gently and incubated for 40min on ice. Cells were pelleted, washed once in SB and resuspended in 1ml 1% PFA supplemented with 0.05% Tween20 and incubated for 30min at RT followed by overnight incubation at 4°C. Cells were pelleted, washed once in SB and then resuspended in 1ml of 0.15M NaCl, 4.2mM MgCl₂ supplemented with 100Kunitz DNaseI (Sigma) for digestion of DNA. After 30min incubation at 37°C cells were pelleted and incubated for 20min on ice in 40µl SB with 10% rat- and 10% mouse serum (Equitech-bio, Inc) to block unspecific binding. 50µl SB with anti-BrdU Alexa Flour 647 Ab and any Abs for intracellular staining were added and incubated overnight at 4°C. Cells were washed twice in SB and analyzed at the LSRII cytometer.

**EdU/ BrdU double detection by multicolor flow cytometry**

1x10^7 red blood cell depleted splenocytes were incubated with anti-CD16/CD32 Abs and EMA in SB for 15min on ice followed by 10min exposure to fluorescent light. Cells were washed and surface-stained with anti-CD38 Alexa Fluor 680 Ab and anti-IgG1 V450 Ab for 30min at 4°C. Cells were washed once in SB followed by an additional washing step in 1xPBS supplemented with 1%BSA. Pellets were resuspended in 120µl fixative (component D of Click-it® EdU Flow cytometry assay kit, Invitrogen) and incubated for 20min at RT and 15min at 4°C. 3ml PBS/BSA were added and cells were pelleted. After one additional washing step in PBS/BSA cells were
resuspended in 2ml Perm/wash buffer (PW) of the click-it® EdU Flow cytometry assay kit and incubated for 5min at RT. Cells were pelleted and resuspended at a final volume of 80µl in PW. After 10min incubation at RT 400µl of the click-it reaction (prepared according to the manufacturers instructions) was added to the cells. After 40min incubation at RT cells were washed twice in PW followed by 2 additional washing steps in PBS/BSA and 2x SB. Cells were stained with NIP-PE and anti-CD95 PE-Cy7 Ab in SB for 30min at 4°C and then washed with 3ml SB. Cell pellets were resuspended in 500µl cold 0.15M NaCl and 1.2ml of pre-cooled 100% ethanol were dropwise added while vortexing gently and incubated for 40min on ice. Cells were pelleted, washed once in SB and resuspended in 1ml of 0.15M NaCl, 4.2mM MgCl₂ supplemented with 100Kunitz DNaseI. After 30min incubation at 37°C cells were pelleted and incubated for 20min on ice in 45µl SB with 10% rat and 10% mouse serum. 60µl SB with anti-B220 APC-Cy7 Ab and anti-BrdU Alexa Fluor 647 Ab were added and incubated overnight at 4°C. Cells were washed twice in SB and analyzed at the LSRII cytometer.

**Gene expression profiling**

mRNA was isolated from 3x10⁵ FACS Aria sorted cell with an RNeasy Micro kit (Qiagen). Biotinylated cRNA was generated with the Illumina TotalPrep RNA Amplification Kit (Life Technologies) and was hybridized to Illumina MouseWG-6 v2.0 Expression BeadChip arrays at the Yale Keck Microarray Facility. Data were analyzed with packages in software of the R project for statistical computing. Raw expression data were normalized by the quantile method provided by the lumi package in R/Bioconductor. Genes with different expression in the early versus late GC subset were defined by two criteria: an absolute difference in expression of ≥1.7, and a statistically significant change in expression as determined by false-discovery rate q < 0.05
(Alan Dabney and John D. Storey). qvalue: Q-value estimation for false discovery rate control. R package version 1.38.0.)
SUPPLEMENTAL REFERENCES


