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CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype

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Memory B cells (MBCs) are long-lived sources of rapid, isotype-switched secondary antibody-forming cell (AFC) responses. Whether MBCs homogeneously retain the ability to self-renew and terminally differentiate or if these functions are compartmentalized into MBC subsets has remained unclear. It has been suggested that antibody isotype controls MBC differentiation upon restimulation. Here we demonstrate that subcategorizing MBCs on the basis of their expression of CD80 and PD-L2, independently of isotype, identified MBC subsets with distinct functions upon rechallenge. CD80$^+$PD-L2$^+$ MBCs differentiated rapidly into AFCs but did not generate germinal centers (GCs); conversely, CD80$^-$PD-L2$^-$ MBCs generated few early AFCs but robustly seeded GCs. The gene-expression patterns of the subsets supported both the identity and function of these distinct MBC types. Hence, the differentiation and regeneration of MBCs are compartmentalized.

Memory B cells (MBCs) provide protection against the consequences of reexposure to antigen[1–3]. These cells can differentiate into antibody-forming cells (AFCs) and make new antibodies or can enter germinal centers (GCs) and provide a renewed source of lasting B cell immunity. Despite the importance of MBCs for vaccine- and infection-induced protection[4–6], there is limited understanding of the nature of these cells and how they participate in secondary responses.

Expression microarray comparisons of MBCs and naive B cells have identified several surface proteins, including CD80, PD-L2 and CD73, that are expressed exclusively on MBCs and serve to categorize MBCs into various phenotypic subsets[7]. Some subpopulations of MBCs are defined by their expression of CD80 and PD-L2, which are both members of the costimulatory and coinhibitory B7 family of surface proteins. These subsets differ in various properties: CD80$^-$PD-L2$^-$ 'double-negative' (DN) MBCs have relatively very few mutations[7,8]; CD80$^+$PD-L2$^+$ 'double-positive' (DP) MBCs have the most mutations; and CD80$^-$PD-L2$^+$ 'single-positive' (SP) MBCs have an intermediate mutational content[7,8]. Although all subsets include cells that express surface B cell antigen receptors (BCRs) of the immunoglobulin M (IgM) or switched IgG isotype, the DN subset is predominantly IgM$^+$, and the SP and DP populations contain progressively more IgG$^+$ cells. These two features, mutation and isotype switching, which are both irreversible DNA alterations that occur during the primary response, indicate that the memory populations are stable and that cells do not move from one population to another (otherwise mutational content and switching would equalize between the populations).

Classically, B cell secondary responses generate rapid effector function, most probably by quickly converting MBCs to AFCs[9]. This raises the question of how the memory compartment undergoes self-renewal in the face of the terminal differentiation of MBCs into AFCs. Although it is unclear how MBCs are homeostatically maintained, gene-expression signatures of stem cells have been identified in MBCs[10–12]. It has been proposed that self-renewing MBCs represent a discrete population that can differentiate into both plasma cells and GC B cells after reexposure to antigen[10,11]. If this were the case, it is possible that all MBCs retain self-renewal potential as well as terminal differentiation potential, with the fate of the cell being determined by environmental cues[13]. Alternatively, these functions may be segregated into different dedicated subsets of MBCs, which may be pre-programmed to respond differently even after encountering identical stimuli.

Two groups have suggested that the MBC pool is functionally subcategorized by antibody isotype expression, either IgM or switched IgG[14,15]. They have found that isotype-switched MBCs differentiate into AFCs, while IgM$^+$ MBCs generate new GCs. From these results they have proposed that surface isotype reflects fundamental differences in MBC potential and have suggested that signaling differences between IgG$^+$ cells and IgM$^+$ cells could govern different functional responses[16,17]. On a parallel track, subsets defined by their expression of CD80 and PD-L2 have been proposed to represent a spectrum of MBC commitment, with the DN cells being more 'naive-like' and the DP cells being more 'memory-like'[9]. Expression of these subset markers on mouse MBCs has been confirmed in various systems[17–20].

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We hypothesized that upon reactivation, the more memory-like DP MBCs would differentiate quickly into effector cells that function by providing new AFCs and not GCs, and that the more naive-like DN MBCs would make new GCs and thus renew the memory pool by providing a new source of cellular immunity.

Here we have tested our hypotheses by examining the function, after reactivation *in vivo*, of MBC populations distinguished by expression of CD80 and PD-L2, while controlling for isotype expression. We generated, purified and transferred these MBC subsets with and without T cells and assessed their ability to make AFCs and GCs upon reexposure to antigen. We found substantial functional heterogeneity that was independent of isotype but was dependent on subset markers. Hence, the functional heterogeneity of MBCs is not determined by BCR isotype, as previously thought, but instead is determined by cell-intrinsic features that are captured by the expression of key surface markers. This view of the composition of the MBC compartment has implications for the monitoring of immune states and thus for vaccine development.

RESULTS

Generation, purification and testing of MBC subsets

Wild-type mice generate exceedingly small populations of MBCs (2×10^4 to 4×10^4 MBCs per spleen)[21,22]. Although such mice develop the MBC subpopulations under investigation[19], there are too few MBCs in wild-type mice to permit purification and subsequent retransfer. Thus, to generate more robust numbers of MBCs, we used a transfer system similar to a published system[7] based on the B1-8 knock-in mouse in which *Igh* is targeted[23]. The B cells that express the immunoglobulin λ-chain in such mice recognize the haptens NP (4-hydroxy-3-nitrophenyl acetyl) and NIP (4-hydroxy-5-iodo-3-nitrophenyl acetyl). To generate MBCs, we transferred 1×10^6 NIP-binding (NIP^+) B cells from BALB/c mice heterozygous for the B1-8 knock-in mutation and heterozygous for deficiency in the κ-chain joining region (J_κ) into BALB/c recipient mice with transgenic expression of an irrelevant (AM14) BCR and with knock-in targeting of the gene encoding a κ-chain variable region of the $V_\kappa 8$ family ($V_\kappa 8R$) (called 'AM14-Tg × $V_\kappa 8R$-KI BALB/c mice' here; **Supplementary Fig. 1**). These recipients have normal lymphoid architecture and composition, but only transferred B cells can respond to NP, since the recipient mice have no NP-specific B cells. We immunized recipients with the T cell–dependent antigen NP–chicken γ-globulin (CGG) precipitated in alum. With the assumption that <5% of transferred cells stably home to the spleen, this would give a precursor frequency before immunization of about 5×10^4 cells, a number we confirmed to be in a range similar to that seen in wild-type mice[7].

At 8 weeks after immunization, ~1–3% of the cells in the recipient spleen had bound to NIP (**Fig. 1a** and data not shown) but were not of the GC phenotype (**Supplementary Fig. 2**); these cells were presumptive MBCs. Approximately 20–30% of the NIP^+ MBCs generated were $IgG1^+$ (**Fig. 1a** and data not shown). We found no NIP^+ MBCs in spleens of immunized recipients not given transfer of NIP^+ B cells (**Fig. 1b**). We observed that these subsets were stable at 26 weeks after immunization (**Supplementary Fig. 3**).

Identity of MBC subsets

To functionally define MBC subsets, we sorted cells by flow cytometry from each subset, along with naive precursor cells for subsequent retransfer or *in vitro* analysis. For retransfer, we purified MBC subsets either by gating on $IgG1^-$ MBCs for DP, SP and DN expression patterns or in separate sorts by gating on IgM^- MBCs for DP and SP expression patterns (**Fig. 1c**). We used these strategies to avoid engaging the BCR of the sorted cells. Separately, we found nearly all of $IgG1^-$ MBCs were IgM^+ and, conversely, that the IgM^- MBCs were mostly $IgG1^+$ (**Supplementary Fig. 4a**). Critically for interpretation of the subsequent experiments, the only types of MBCs that could generate the $IgG1^+$ AFCs assayed in our study were those that expressed IgM, IgG1 or IgG3; however, $IgG3^+$ cells represented only ~2% of the MBCs (**Supplementary Fig. 4b**).

Initially, to better define subset identities, we did microarray-based transcriptome analysis of cells sorted as described above but without consideration of BCR isotype. All three MBC subsets were transcriptionally more similar to each other than they were to naive cells (**Supplementary Fig. 5a**). However, MBC subsets defined by their expression of CD80 and PD-L2 had distinct gene-expression patterns (**Supplementary Fig. 5b**). Notably, genes encoding several transcription factors that are candidates for specification of subset identity, including *Mef2b*, *Uhrf1*, *Zbtb32*, *Bcl6*, *Satb1* and *Klf2*, were expressed differently by the MBC subsets. While the functional relevance of the specifically expressed genes remains to be determined, these data provided further support for exploring the overall functional abilities of these cell types.

Functional differences among subsets upon immunization

To assess the function of MBC subsets upon secondary immunization, we sorted MBCs and transferred 5×10^4 of the purified MBCs into recipient mice with expression of the irrelevant AM14 BCR targeted to

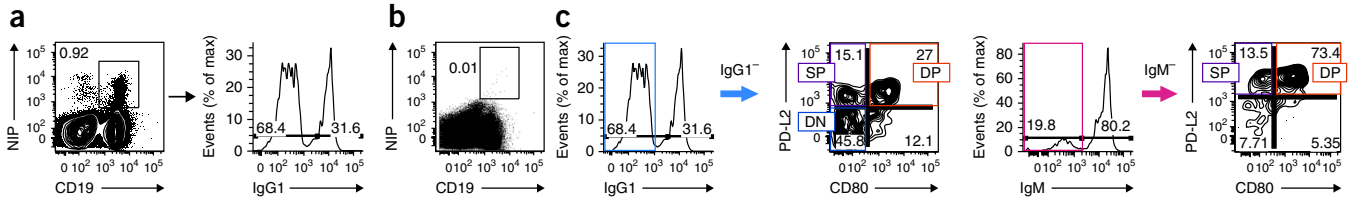


Figure 1 Generation and purification of MBC subsets. (**a**) Flow cytometry of splenic cells from AM14-Tg × $V_\kappa 8R$-KI recipient mice given NP-specific B cells and immunized with NP-CGG in alum, assessed 8 weeks later (left), and staining of IgG1 on the gated cells outlined at left (right). Number adjacent to outlined area (left plot) indicates percent $CD19^+NIP^+$ antigen-specific B cells among live cells; numbers above bracketed lines (right plot) indicate percent $IgG1^-$ cells (left) or $IgG1^+$ cells (right) among those $CD19^+NIP^+$ cells. (**b**) Flow cytometry of splenic B cells from AM14-Tg × $V_\kappa 8R$-KI mice immunized with NP-CGG in alum without transfer of NP-specific B cells, assessed 8 weeks later as in **a**. (**c**) Flow cytometry of MBCs gated as in **a** and stained for IgG1 (left half) or IgM (right half), followed by the identification of $CD80^+$PD-$L2^+$ DP, $CD80^-$PD-$L2^+$ SP and $CD80^-$PD-$L2^-$ DN subsets among $CD19^+NIP^+$ $IgG1^-$ MBCs (blue outlined area, far left) or $CD19^+NIP^+$ IgM^- MBCs (magenta outlined area, middle right). Numbers above bracketed lines as in **a** (far left) or percent IgM^- cells (left) or IgM^+ cells (right) (middle right plot); numbers in quadrants (middle left and far right) indicate percent cells in each. Data are from one experiment with one mouse representative of nine independent experiments with 20–30 mice per experiment (**a**), three independent experiments with three mice per experiment (**b**) or nine independent experiments with 20–30 mice per experiment (**c**, left) or two independent experiments with 43–45 mice per experiment (**c**, right).

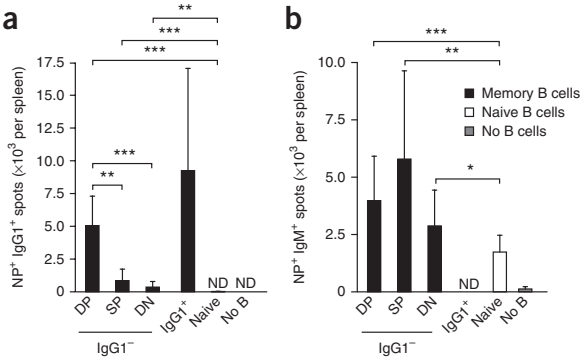
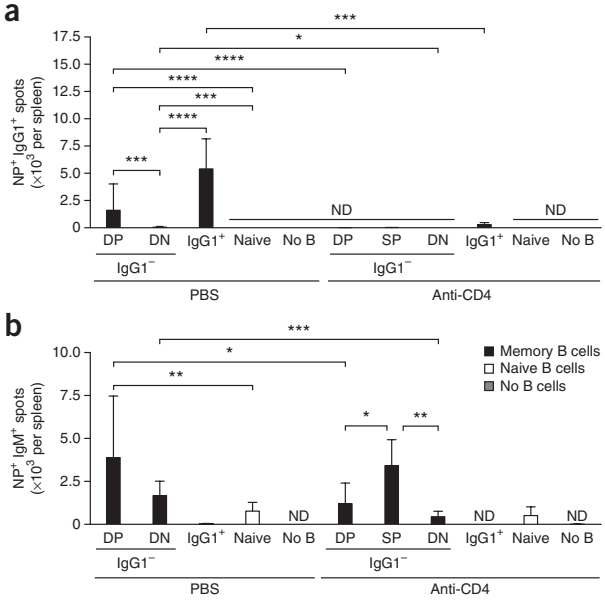


Figure 2 DP IgG1$^-$ and IgG1$^+$ MBCs are the main producers of early IgG1$^+$ AFCs. Enzyme-linked immunospot (ELISPOT) assay of NP$^+$ IgG1$^+$ AFCs (**a**) and IgM$^+$ AFCs (**b**) in recipient mice given transfer of DP, SP or DN IgG1$^-$ MBCs, IgG1$^+$ MBCs, naive B cells (Naive) or no B cells (No B), assessed 3.5 d after immunization of the host mice with NP-OVA in alum. ND, not detected. $^*P < 0.05$, $^{**}P < 0.001$ and $^{***}P < 0.0001$ (Mann-Whitney nonparametric, two-tailed test). Data are pooled from two independent experiments with 5–14 (**a**) or 3–16 (**b**) mice per group (error bars, s.d.).

Igh and the expression of V_κ8R noted above (called 'AM14-KI × V_κ8R-KI BALB/c mice' here), which allowed comparison of the function of MBC subsets in different mice without confounding host effects. Given the ~5% recovery of transferred B cells, the precursor frequency of the responding cells was at or below what would be expected in an intact wild-type mouse. Of note, the number of mutations in the gene encoding the immunoglobulin λ-chain V region, per B cell, was low in all of these populations, consistent with limited affinity maturation in most MBCs (compared with that of long-lived plasma cells that contain many more V-region mutations)[24] (F.J.W. and M.J.S., data not shown). In particular, all populations had MBCs that were not mutated[7,24], and notably more than half of the DP B cells had no replacement mutations throughout their light-chain V regions, and 70% lacked any replacement mutations in complementarity-determining regions, where such changes might affect affinity (**Supplementary Fig. 6**). This finding suggested that the starting affinities of MBC populations would be relatively similar.

We immunized recipient mice with NP-ovalbumin (OVA) precipitated in alum at 1 d after transfer of MBCs (**Supplementary Fig. 7a**).



In some groups, we also included 'rested effector' DO11.10 T cells, which had been stimulated and allowed to rest *in vitro*; these have memory cell properties and thus are a physiological partner for MBCs[4]. DO11.10 T cells have transgenic expression of an OVA-specific T cell antigen receptor (TCR), and we obtained these cells from DO11.10 TCRα-deficient (*Tcra*$^{-/-}$) BALB/c mice. They had a CD25$^-$PD-1$^-$CD44$^+$ surface phenotype (**Supplementary Fig. 7b**), similar to the reported phenotype of memory T cells[4]. We call these cells simply 'memory T cells' here. Such cells represent a source of homogeneous and synchronous memory T cells that could not have been generated via *in vivo* priming, as this generated inadequate numbers of recoverable cells (data not shown).

Early burst of AFCs generated by IgG1$^-$ DP MBCs and IgG1$^+$ MBCs

We assessed the ability of IgG1$^-$ or IgG1$^+$ MBC subsets in the spleen to differentiate into AFCs at 3.5 d after immunization. Of note, DP IgG1$^-$ MBCs produced numbers of IgG1$^+$ AFCs similar to those produced by the total population of IgG1$^+$ MBCs (**Fig. 2a**). Hence, at least one MBC subset composed almost completely of IgM$^+$ MBCs was just as efficient a source of isotype-switched AFCs as previously switched MBCs. Among IgG1$^-$ MBCs, the DP subset generated 5- and 13-fold more NP$^+$ IgG1$^+$ AFCs than did SP B cells or DN B cells, respectively (**Fig. 2a**), which indicated that these memory-like MBCs had a greater capacity to rapidly undergo isotype switching and to differentiate into AFCs. DP MBCs also generated AFCs with the highest relative affinity of any subset of MBCs (**Supplementary Fig. 8**).

At day 3.5, IgG1$^-$ DN MBCs still produced more IgG1$^+$ AFCs than did naive B cells (**Fig. 2a**). IgM$^+$ NP-specific AFCs were generated to the same extent among IgG1$^-$ MBC subsets, with the exception of IgG1$^-$ SP MBCs, which generated slightly more AFCs (**Fig. 2b**). As with IgG1$^+$ AFCs, all three MBC subsets also generated more IgM$^+$ AFCs than did naive B cells (**Fig. 2b**). Hence, in terms of the generation of IgG1$^+$ AFCs, even DN MBCs, which were inferior to other MBCs, were still much more capable than naive B cells.

IgM$^+$ MBCs do not depend on memory T cells to make AFCs

In the experiments reported above, MBCs were transferred together with antigen-specific memory T cells. However, to establish the degree to which MBC responses depend on such T cells, we repeated those experiments without adding T cells. We found that IgG1$^-$ DP MBCs generated more IgG1$^+$ AFCs than DN MBCs did, even in the absence of a preexisting memory T cell population (**Fig. 3a**). Nonetheless, IgG1$^-$ DN MBCs generated fewer IgG1$^+$ AFCs (one sixth the frequency) and IgM$^+$ AFCs (roughly half the frequency) when only help from naive T cells of the recipient was available (**Fig. 3**) than in experiments in which memory T cells were also transferred (**Fig. 2**). These data indicated that IgG1$^-$ MBCs needed specific T cells for optimal population expansion and/or differentiation into IgG1$^+$ AFCs but that such T cells did not alter the underlying nature of the responses of the MBC subsets.

Figure 3 Requirement for MBCs in the generation of early AFC responses by T cells. ELISPOT analysis of NP$^+$ IgG1$^+$ AFCs (**a**) and NP$^+$ IgM$^+$ AFCs (**b**) in spleens of recipient mice treated with PBS (n = 6–19 mice per group) or anti-CD4 (n = 5–13 mice per group), then given transfer of DP, SP or DN IgG1$^-$ MBCs, IgG1$^+$ MBCs, naive B cells or no B cells and immunized with NP-OVA in alum, assessed 3.5 d later. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ (Mann-Whitney nonparametric, two-tailed test). Data are pooled from two (anti-CD4) or three (PBS) independent experiments (error bars, s.d.).

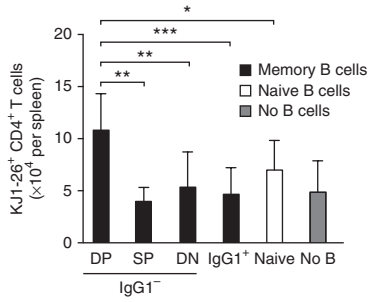


Figure 4 DP IgG1$^-$ MBCs promote more population expansion of cognate T cells than do other B cell types. Quantification of KJ1-26$^+$ CD4$^+$ OVA-specific T cells in mice that received memory T cells along with DP, SP or DN IgG1$^-$ MBCs; IgG1$^+$ MBCs, naive B cells or no B cells, assessed 3.5 d after immunization of the host mice with NP-OVA in alum. $^*P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$ (two-tailed *t*-test). Data are pooled from two independent experiments with 6–19 mice per group (error bars, s.d.).

T cell requirement for MBCs to generate AFCs

To determine whether MBC responses required any type of T cell, we pretreated recipient mice with monoclonal antibody to CD4 to achieve depletion of recipient T cells before transferring MBC subsets (**Supplementary Fig. 9**). Almost no IgG1$^+$ AFCs were generated from any IgG1$^-$ MBCs in recipients depleted of T cells (**Fig. 3a**), which indicated that some form of T cell help was required for IgM$^+$ MBCs to rapidly make switched antibodies. IgG1$^+$ MBCs generated 18-fold more NP$^+$ IgG1$^+$ AFCs in recipients that had naive T cells than in recipients depleted of T cells (**Fig. 3a**). These findings indicated that even IgG$^+$ MBCs greatly, although not absolutely, depended on T cells to generate IgG1$^+$ AFCs.

Furthermore, IgG1$^-$ DP MBCs generated threefold more IgM$^+$ AFCs and IgG1$^-$ DN MBCs generated 3.7-fold more IgM$^+$ AFCs in intact recipients than in recipients depleted of T cells (**Fig. 3b**). Due to the limited numbers of IgG1$^-$ SP MBCs sorted, we were able to study this subset only in recipients that were depleted of T cells or that had received memory T cells: IgG1$^-$ SP MBCs generated 1.7-fold more IgM$^+$ AFCs with memory T cells (**Fig. 2b**) than without T cells (**Fig. 3b**). IgG1$^-$ SP MBCs were able to make the most IgM$^+$ AFCs in either recipient. These observations indicated that IgG1$^-$ MBCs did not require T cells to differentiate into IgM$^+$ AFCs. However, T cells contributed to the numerical expansion of IgM$^+$ AFCs and generation of IgG1$^+$ AFCs, although such T cell help could derive from either polyclonal naive T cells or memory antigen-specific T cells.

Memory T cell populations expand more with IgG1$^-$ DP MBCs

Both CD80 and PD-L2, used to define MBC subsets, directly interact with molecules expressed on helper T cells[25–27]. Therefore, we sought to determine if MBC subsets had different effects on antigen-specific memory-like T cells. Indeed, OVA-specific T cells identified as KJ-126$^+$ CD4$^+$ T cells proliferated to a significantly greater extent in recipients that received IgG1$^-$ DP MBCs than in recipients that received other MBC types or naive B cells (**Fig. 4**). The finding that DP MBCs were superior in this context might indicate that expression of both members of the B7 family could contribute to such an outcome, although this remains to be tested directly.

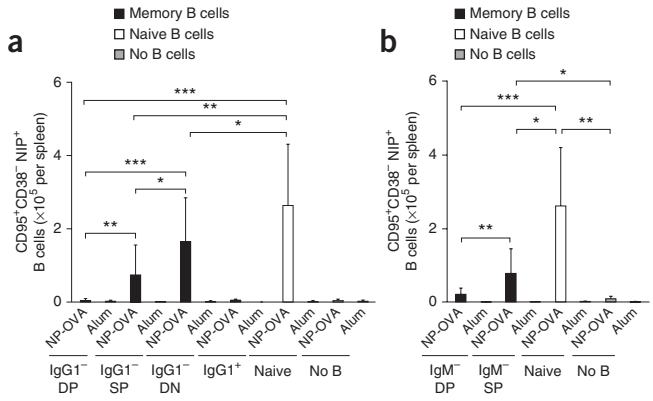


Figure 5 GC B cells are generated from SP or DN IgG1$^-$ MBCs or naive B cells but not from DP MBCs. Quantification of CD95$^+$CD38$^-$ NIP$^+$ GC B cells in recipient mice given transfer of memory T cells along with DP, SP or DN IgG1$^-$ MBCs, IgG1$^+$ MBCs, naive B cells or no B cells (**a**) or DP or SP IgM$^-$ MBCs, naive B cells or no B cells (**b**), assessed 10.5 d after immunization of host mice with NP-OVA in alum or alum alone. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.0001$ (two-tailed *t*-test). Data are pooled from three independent experiments with 8–22 mice per group (NP-OVA) or 1–15 mice per group (alum) (**a**) or two independent experiments with 6–13 mice per group (NP-OVA) or 2–6 mice per group (alum) (**b**; error bars, s.d.).

DN and SP MBCs, but not DP MBCs, generate new GCs

Published studies have suggested that only IgM$^+$ MBCs generate new GCs[14,15]. However, those studies did not separate MBCs according to subset as defined by CD80 and PD-L2. Given the heterogeneity we observed among IgM$^+$ MBCs, we hypothesized that they might also differ in their capacity to make GCs upon reimmunization. To test this, we transferred different subsets of IgG1$^-$ MBCs plus OVA-specific memory T cells into recipient mice and immunized the recipients with NP-OVA in alum, then obtained cells from the recipients 10.5 d after immunization. IgG1$^-$ DP MBCs did not generate GC B cells, while IgG1$^-$ DN MBCs produced almost as many GC B cells as did naive B cells (**Fig. 5a**). Conversely, we assessed IgM$^-$ MBCs, which had previously been thought to be unable to generate secondary GCs. In this case, DN IgM$^-$ MBCs were too rare to study, but we were able to test SP MBCs and DP MBCs. IgM$^-$ SP MBCs were able to generate GC B cells, and such cells were similar in number to those derived from IgG1$^-$ SP MBCs (**Fig. 5b**). These results indicated that the capacity of MBCs to make GCs was not dependent specifically on their isotype, since SP MBCs of either isotype and IgM$^+$ DN MBCs were able to make GCs with similar efficiency. Instead, subset identity was a better predictor of the GC-forming capacity of MBCs.

DN MBCs make the most late-appearing AFCs

At day 10.5 after transfer of MBCs and immunization of recipients (as in **Fig. 2**), in contrast to results obtained at day 3.5, IgG1$^-$ DN MBCs contributed most of the IgG1$^+$ NP-specific AFCs in the spleen (**Fig. 6a**). IgG1$^-$ DN MBCs generated sevenfold more IgG1$^+$ AFCs than did IgG1$^-$ DP MBCs and generated 2.5-fold more AFCs than even IgG1$^+$ MBCs did at day 10.5 (**Fig. 6a**). Since GCs were readily generated by IgG1$^-$ DN MBCs (**Fig. 5a**), this finding suggested that AFCs derived from IgG1$^-$ DN MBCs could emanate from secondary GCs. In keeping with that observation, IgG1$^-$ DN MBCs and naive B cells, which both generated the largest numbers of NP$^+$ GC B cells, also generated the most IgG1$^+$ AFCs at 10.5 d after immunization (**Fig. 6a**). At day 10.5, both DP and SP subsets of IgM$^-$ MBCs generated fewer IgG1$^+$ AFCs than did naive B cells or IgG1$^-$ DN MBCs (**Fig. 6a**). There were few IgM$^+$ AFCs in the spleen at day 10.5, with little difference in the numbers of AFCs generated by any of the IgG1$^-$ MBC subsets (**Fig. 6b**). Overall, MBC subsets that generated an early large burst of AFCs did not proliferate further and were overtaken by progeny of

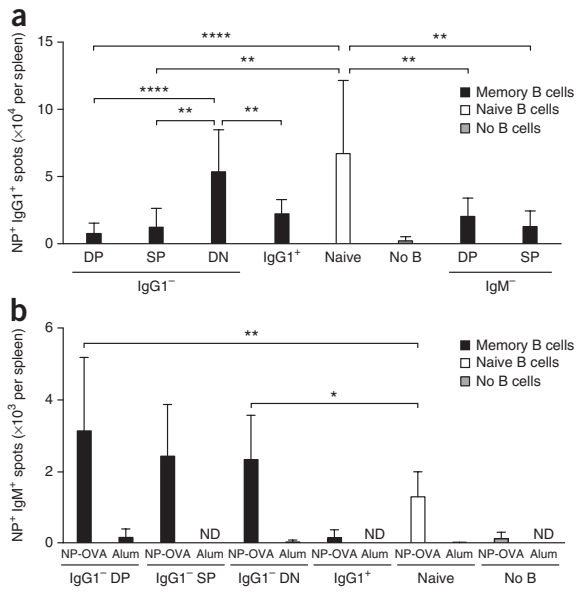


Figure 6 Late formation of IgG1$^+$ AFCs is dominated by DN IgG1$^-$ MBCs and naive B cells, but that of IgM$^+$ AFCs is not. (**a**) ELISPOT analysis of NP$^+$ IgG1$^+$ AFCs in the spleen of recipient mice given transfer of memory T cells along with DP, SP or DN IgG1$^-$ MBCs, IgG1$^+$ MBCs, naive B cells, no B cells or DP or SP IgM$^-$ MBCs, assessed 10.5 d after immunization of host mice with NP-OVA in alum (results after immunization with alum not presented here as this produced no detectable responses). (**b**) ELISPOT analysis of NP$^+$ IgM$^+$ AFCs in the spleen of recipient mice given transfer of memory T cells plus DP, SP or DN IgG1$^-$ MBCs, IgG1$^+$ MBCs, naive B cells, no B cells, assessed 10.5 d after immunization of host mice with NP-OVA in alum or alum alone. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ (Mann Whitney nonparametric, two-tailed test). Data are pooled from four independent experiments with 7–24 mice per group (**a**) or two independent experiments with 6–16 mice per group (NP-OVA) or 1–11 mice per group (alum) (**b**; error bars, s.d.).

MBC subsets that were more capable of proliferation and secondary GC generation.

Gene-expression patterns of MBC subsets

The data reported above demonstrated that DP MBCs differentiated rapidly into AFCs, whereas DN MBCs underwent a substantial early proliferative burst, then differentiated into GC B cells. We sought clues to such activities in microarray data as described above. Notably, the transcriptome of DN MBCs demonstrated stronger signatures of genes encoding cell cycle–promoting molecules than did that of naive B cells, whereas this was not the case for DP MBCs. This was based on cell-cycle annotation according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (**Fig. 7**), as well as several related pathways defined by the Reactome Pathway Browser, including E2F-regulated genes[28] (data not shown). To further bolster the conclusion that genes encoding cell cycle–related molecules had higher expression in DN cells than in DP cells, we used quantitative PCR analysis of sorted MBC subsets obtained 45 weeks after immunization (as in **Supplementary Fig. 1**). We confirmed that three of five genes from the KEGG cell-cycle list that we tested here (*Cdc20*, *Mcm5* and *Plk1*, but not *E2f* or *Ccn2*) had higher expression in DN cells

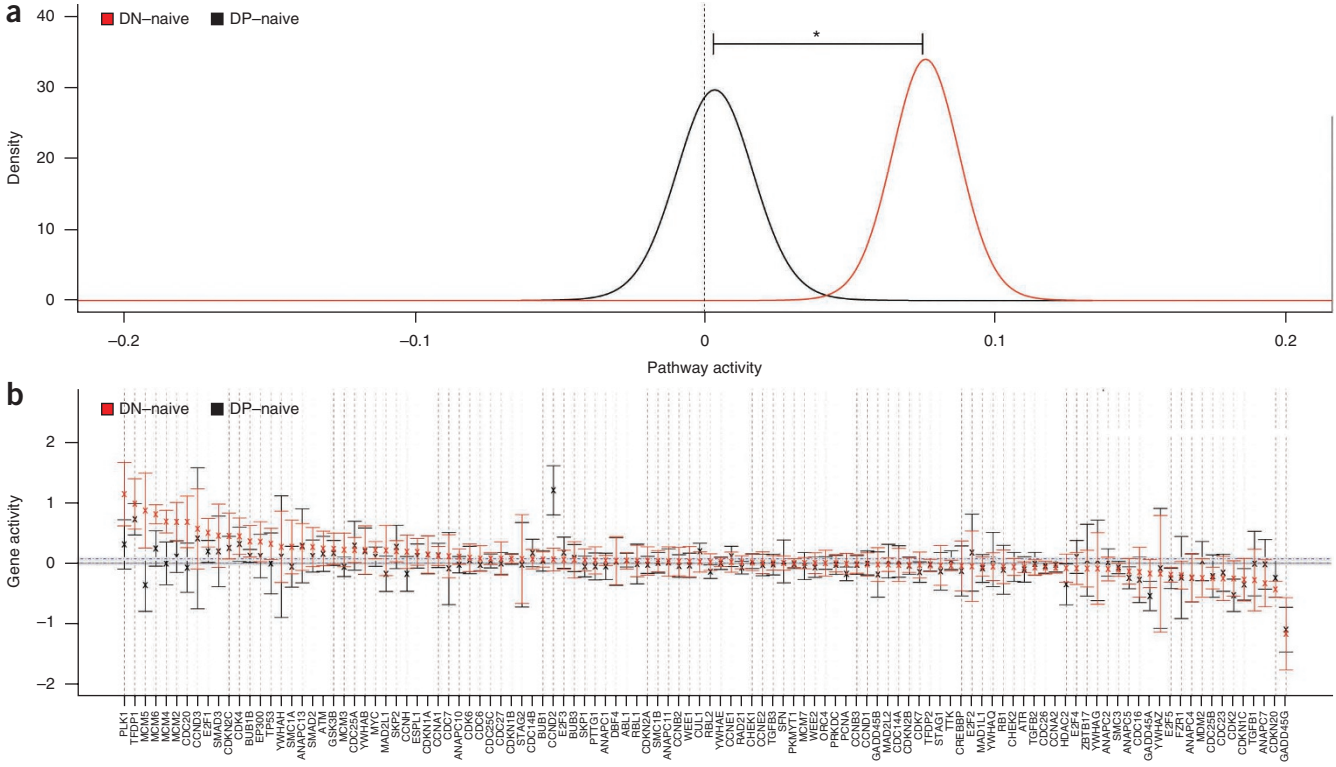


Figure 7 Significant activation of the cell-cycle pathway in the DN subset. (**a**) Activity of the KEGG cell-cycle gene set in DN and DP MBCs, assessed with QuSAGE software ('quantitative set analysis of gene expression') and presented as probability density function results for the DN subset relative to those of naive B cells (DN-naive) and for the DP subset relative to those of naive B cells (DP-naive). Dashed vertical line indicates 'neutral' pathway activity. $^*P = 0.001$. (**b**) Gene activity of the products encoded in the KEGG cell-cycle pathway in the DN subset and DP subset, presented as the $\log_2$ difference in expression in DN or DP cells relative to that in naive B cells. Data are from one experiment with three biological replicates per cell type (mean values with 95% confidence interval (error bars) in **b**).

than in DP cells in this assay (**Supplementary Fig. 10** and data not shown). The transcriptional repressor Zbtb32, which is associated with plasma cell differentiation[29], had over tenfold higher expression in DP MBCs than in DN MBCs, as determined by quantitative PCR (**Supplementary Fig. 9**). Together these data provide initial clues as to why DN MBCs might be more prone to proliferate rather than differentiate, whereas DP MBCs are destined for AFC differentiation upon reimmunization.

DISCUSSION

In this study we have elucidated the distinct functions of defined, stable MBC subsets described before[7,8,17–20,30]. Studying these three types of MBCs, we found that there was a spectrum of activity ranging from more 'naive-like' to more 'memory-like'. DN MBCs, which phenotypically resembled naive B cells, were the MBCs able to form substantial secondary GC responses with efficiency similar to that of naive B cells. Notably, they also differed from naive B cells, since DN IgM$^+$ MBCs were able to generate switched AFCs at critical early time points, but naive B cells were not. In contrast, DP IgM$^+$ MBCs produced no GC B cells but generated notably quick and large isotype-switched secondary AFC responses. SP MBCs had intermediate properties, with some ability to make secondary GCs and a robust ability to spawn AFCs, albeit to a lesser degree than DP MBCs.

This work should revise current views on the heterogeneity of the MBC compartment. It has been posited that expression of IgM and IgG itself defines functional MBC subsets[14,15]. However, our data have indicated that these are surrogate markers, although they correlate to some degree with function. This conclusion is substantiated by our demonstration of functional heterogeneity in both the IgM$^+$ MBC compartment and IgG1$^+$ MBC compartment, in which the markers CD80 and PD-L2 delineated unique functional abilities. Conversely, across IgM$^+$ and IgG1$^+$ phenotypes, MBCs with similar expression profiles for members of the B7 family had similar functions, as most clearly highlighted by the equal GC-forming capacity of SP MBCs regardless of isotype.

Nonetheless, the earlier findings[14,15] can be reconciled with our present observations. IgM$^+$ MBCs have been reported to generate secondary GCs. In agreement with that finding, we found that IgM$^+$ MBCs did include a substantial fraction of DN MBCs that were able to reenter GCs. However, among IgM$^+$ MBCs were both SP MBCs and DP MBCs, which generated a rapid isotype-switched AFC response, with DP MBCs generating essentially no secondary GC response. Hence, the responses of IgM$^+$ MBCs were truly heterogeneous and expression of IgM by itself was not predictive of MBC activity upon restimulation. Similarly, IgG$^+$ MBCs are reported to make only AFCs. However, we found that IgG1$^+$ MBCs were also heterogeneous in their expression of CD80 and PD-L2. The activity of SP and DP IgG1$^+$ MBC subsets paralleled that of the similar IgM$^+$ MBC subsets, most notably in the ability of SP cells to make secondary GC responses. These findings elucidate unsuspected functional heterogeneity, even among IgG1$^+$ MBCs.

Subsets of MBCs also express CD73 (refs. 7,12). From these data we infer that there could be as many as five different MBC subsets defined only by their expression of CD80, PD-L2 and CD73, notwithstanding diversity of isotype expression. Thus, there could be yet more functional heterogeneity to be discovered.

While our work here focused on the intrinsic function of MBCs, we also studied the effects of T cells on MBCs and of MBCs on T cells. A strength of our study involved the use of memory T cells, which normally would be the collaborating partner for MBCs; thus, we believe our results probably reflect the physiological condition. The addition of T cells, and in particular 'rested effector' cells that are qualitatively similar to memory T cells, greatly augmented the responses of all subsets and allowed the clearest demonstration of the subset differences. Notably, the nature of the differences between the subsets was consistent regardless of the T cells being used. In contrast, when we depleted mice of all T cells, including polyclonal host T cells, there were minimal MBC responses, except by IgG1$^+$ MBCs, which generated some (although substantially fewer) AFCs. This result was in contrast to responses to those obtained for virus-like particles, which do not require T cells for MBC responses[22].

Another advantage of using defined T cell populations to act together with MBCs was that we were able to track T cell responses to immunization in the context of different types of MBCs. One notable finding was that populations of antigen-specific T cells expanded to a greater degree when they acted together with DP MBCs. This occurred despite the fact that DP MBCs themselves actually do not form robust GCs. We speculate that expression of CD80 and PD-L2 modulated T cell responses, since T cells should express the receptors for both ligands (i.e., CD28, CTLA-4 and PD-1)[31,32]. This finding would be reminiscent of the effects on follicular helper T cells of the expression of these B7 family members on B cells in a primary GC reaction[27,33]. Our results indicate an added layer of complexity and 'tuning' in T cell–B cell interactions during secondary responses.

A notable implication of our findings is that effector function and self-renewal are segregated into two distinct cell types in the MBC compartment. This scenario is unlike the situation for both naive B cells and T cells, in which a single cell has the capacity to form all of the dedicated progeny, including effector and memory type cells. In this context, the MBC compartment adopts a configuration more like a stem-cell and committed-progenitor model[34]. In this view, DN MBCs resemble secondary stem cells, while DP MBCs would correspond to committed progenitor cells. We predict that there might be parallels to well-studied stem-cell systems[35,36]. Indeed, both bulk memory T cells and memory B cells share some gene-expression patterns with hematopoietic stem cells[11]. Unseparated MBCs express *Bmpr1a*[10–12], which encodes a receptor that commonly regulates stem cell maintenance versus differentiation[37]. Segregation of function into different MBC subsets may ensure self-renewal while still enabling robust responses to external signals. This suggests that a layered or multi-subset approach to secondary responses has evolved that may be particularly suited for pathogens that mutate, as the DN subset contains MBCs that can respond to such adaptations, even if the antibody secreted from progeny of DP MBCs has little cross-reactivity with a mutant pathogen[6,38,39].

Additionally, our findings have implications for vaccine design and monitoring. Different pathogens may require different types of MBC responses for effective clearance. Rational vaccine design would involve optimizing adjuvants, routes, timing and composition to elicit the desired MBC subsets that would in turn match the need for effective clearance and immunity to each specific pathogen. Monitoring of vaccine responses would ideally include measurement of antigen-specific B cells in all relevant MBC subsets; at present, serological responses at sometimes arbitrary time points are the only accepted measure of success[2,40]. Incorporation of this new knowledge and approach could shed light on why some vaccines yield protection of limited duration or efficacy. In this context, exactly how pathogens and other immunological contexts (including adjuvants, routes and the molecular structure of the antigen) may influence subset development and even inherent characteristics is not yet known but would be important to determine.

Finally, how the subsets of MBCs we have defined achieve their different functions needs to be resolved. The detailed molecular basis

for such differentiation at the level of transcription factor expression, epigenetic programming and signaling remains to be defined. Nonetheless, as an initial step, our microarray analysis has highlighted differences in the expression of key transcription factors, including members of the Zbtb family that are associated broadly with lineage specification. We have also identified transcriptional signatures related to cell-cycle progression that may underlie the propensity of DN MBCs to undergo initial population expansion rather than differentiation. Our results are an advance in demonstrating differences in the functionality of stable, authentic subsets; notably, expression of CD80 on some human MBCs has already been reported[41]. Our results will help elucidate a rapidly evolving literature on the heterogeneity of MBCs. In particular, they explain how the MBC compartment overall both provides immediate effector function and replenishes itself, which has been a central question in the field.

METHODS

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

Accession codes. GEO: microarray data, GSE51604.

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

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AUTHOR CONTRIBUTIONS

G.V.Z.-C., F.J.W., M.M.T., K.L.G.-J. and M.J.S. designed the research; G.V.Z.-C. and F.J.W. did research; S.S. did the T cell–counting experiment; H.M. and S.H.K. analyzed microarray data; K.L.G.-J. and F.J.W. provided technical support and conceptual advice; and G.V.Z.-C. and M.J.S. analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and immunization. B1-8 knock-in BALB/c mice were generated as described[23] and were maintained on the J_κ-deficient strain[42] to ensure enrichment for λ chain–positive NP-specific B cells. Mice wild-type for the B1-8 knock-in mutation and homozygous for J_κ deficiency were crossed with BALB/c mice from The Jackson Laboratory to generate BALB/c mice heterozygous for the B1.8 mutation and for J_κ deficiency, which were used for naive controls and for the transfer of NP^+ B cells used to generate MBCs.

AM14-Tg × V_κ8R-KI BALB/c mice were generated as described[43–45] and were used as recipient mice for primary immunization, sorting of MBCs and analysis of secondary responses at week 4.

AM14-KI × V_κ8R-KI BALB/c mice were generated as described[45,46] and were used as recipient mice for secondary responses. DO11.10 *Tcra*$^{-/-}$ BALB/c mice were generated as described[47] and were used as a source for T cells for the generation of memory T cells. Their TCR recognizes amino acids 323–339 (ISQAVHAAHAEINEAGR) of OVA. BALB/c mice from The Jackson Laboratory were used as a source of APCs for the generation of memory T cells. All mice were maintained under specific pathogen–free conditions. The Yale Institutional Animal Care and Use Committee approved all animal experiments.

For the generation of MBCs in a primary response, mice were immunized intraperitoneally with 50 μg of NP-CGG precipitated in alum. The ratio of NP moieties to CGG moieties ranged from 26 to 33. For secondary responses mice were immunized intraperitoneally with 50 μg of NP-OVA precipitated in alum. The ratio of NP moieties to CGG moieties ranged from 8 to 10. Precipitated alum alone was also used as a control in day 10.5 experiments. All mice were immunized at 6–12 weeks of age.

Antibodies and detection reagents. The following staining reagents were prepared in the Shlomchik laboratory as described[48]: NIP-binding reagents (NIP-allophycocyanin), monoclonal anti-CD4 (GK1.5), anti-FcγRIII/II (2.4G2), monoclonal anti-IgM (B7-6), anti-CD19 (1D3.2) and anti-CD44 (1M7). Anti-PD-L2 (TY-25), anti-CD80 (16-10A1), anti-CD38 (90), antibody to the DO11.10 TCR (KJ1-26) and anti-CD62L (Mel-14) were from BioLegend. Anti-IgG1 (A85-1) and anti-CD4 (RM4-4) were from BD Biosciences. Anti-CD19 (1D3) and anti-CD95 (Jo2) were from BD Pharmingen.

Flow cytometry, cell sorting and ELISpot assay. Flow cytometry and the ELISPOT assay of AFC formation were done as described[27]. Dead cells were excluded in all flow cytometry experiments through the use of propidium iodide or ethidium monoazide (Invitrogen). Doublets were excluded in the gating strategy.

For analysis of the production of AFCs by ELISPOT assay or antibody by ELISA, plates were coated overnight at 4 °C with 5 μg NP-BSA conjugated at the appropriate ratio (NP_4-BSA for IgM, NP_{16}-BSA for IgG1 affinity studies and $NP_{1.9–2.0}$-BSA for IgG1 affinity studies, where subscripted numbers indicate NP moieties). For sorting of cells by flow cytometry, spleens were pooled from AM14-Tg × V_κ8R-KI BALB/c mice, maintained on ice in buffers without sodium azide and treated with unlabeled anti-FcγRIII/II (2.4G2) and stained with the relevant antibodies (identified above). Cells were sorted on a FACSAria (BD Biosciences). Data were analyzed with FlowJo software (TreeStar).

Adoptive transfer for the generation of memory cells. Splenic B cells from B1-8 mice were prepared with an EasySep Mouse B Cell Enrichment Kit according to the manufacturer's protocol (StemCell Technologies). Single-cell suspensions were transferred intravenously into tail veins of recipient mice. Approximately 1×10^6 NIP^+ B cells were transferred per mouse. The purity of B cells was typically 90%. Approximately 12–24 h after transfer, mice were immunized as described above.

Adoptive transfer for secondary responses. MBC subsets were sorted from AM14-Tg × V_κ8R-KI BALB/c mice or naive B cells were sorted from B1-8 mice as described above. Single-cell suspensions of 5×10^4 B cells and, where noted, 2×10^5 memory T cells were transferred intravenously into tail veins of recipient AM14-KI × V_κ8R-KI BALB/c mice. The purity of sorted cells was above 98%. Approximately 20–24 h after transfer, mice were immunized as described above.

Isolation of naive CD4 T cells and preparation of APCs. Naive $CD4^+$ T cells were purified from DO11.10 *Tcra* $^{-/-}$ BALB/c mice. After lysis of red blood cells, cell suspensions from spleen underwent negatively selection with an EasySep $CD4^+$ Mouse T Cell Enrichment Kit according to the manufacturer's protocol (StemCell Technologies). Resulting cells were routinely >95% $CD4^+$ $KJ\text{-}126^+$.

Splenic APCs from BALB/c mice were prepared by depletion of T cells from BALB/c cell populations for 30 min on ice with anti-Thy-1.2 supernatant (30H12) in PBS and rabbit complement for 30 min at 37 °C, followed by irradiation (2000 cGy).

Generation of memory T cells. $CD4^+$ T cell effector cells were generated *in vitro* as described[4,49]. Splenic APCs from BALB/c mice (prepared as described above; 2×10^5 per ml) were cultured for 4 d with naive $CD4^+$ T cells (2×10^5 per ml) in the presence of 5.6 μM OVA peptide (amino acids 323–339; RP10610; GenScript) and 50 U/ml interleukin-2 for 4 d in Click's medium (a modification of Eagle's essential medium with Hanks' salts, a higher concentration of essential amino acids, nonessential amino acids, sodium pyruvate and precursors of nucleic acids) supplemented with 5% FCS (HyClone), 2 mM L-glutamine, 10 mM HEPES buffer, pH 7, penicillin and streptomycin, 1% nonessential amino acids and 2-mercaptoethanol. After 4 d, effector cells were washed thoroughly and then were recultured for 4 d in fresh Click's medium in the absence of antigens or cytokines. Live cells were isolated by Percoll gradient separation. The resulting population was routinely >95% $CD4^+$ $KJ\text{-}126^+$.

Depletion of $CD4^+$ T cells. For depletion of $CD4^+$ T cells from AM14-KI × V_κ8R-KI BALB/c recipient mice, the GK1.5 monoclonal antibody to CD4 was produced and purified as described[43]. For most experiments, mice were given intraperitoneal injection of 300 μg GK1.5 or PBS once 4 d before transfer of B cells. In one experiment, mice were injected twice 2–4 d before transfer of B cells. The depletion of T cells was monitored by checking of one mouse on the day of transfer and at the time the spleen was collected.

Microarray generation and data analysis. After memory B cell subset samples were sorted by surface expression of CD80 and PD-L2, mRNA was isolated from those cells with an RNeasy Micro kit according to the manufacturer's instructions (Qiagen) 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 DN subset versus the DP subset were defined by two criteria: an absolute $\log_2$ difference in expression of ≥1, and a statistically significant change in expression as determined by the limma software package ('linear models for microarray data') with a Benjamani-Hochberg false-discovery rate cutoff of $q < 0.05$.

Principal-component analysis (PCA) was performed on gene-expression profiles of the 350 probes with the most variable expression, with coefficient of variation greater than 0.05. Gene-set enrichment analyses were performed with QuSAGE software (version 1.3.1)[50] and the n.point parameter was set to 214 for convolution of the probability density function. The KEGG cell-cycle gene set was downloaded from the Molecular Signatures database (MSigDB) version 4.0 (http://www.broadinstitute.org/gsea/msigdb/index.jsp/).

Quantitative PCR. Cell populations were purified by sorting, then total RNA was isolated from the sorted cells with an RNeasy Plus Micro kit (Qiagen, 74034) and first-strand synthesis was performed with the SuperScript III First-Strand Synthesis System for RT-PCR (18080-051; Invitrogen). Quantitative PCR was performed with a SYBR FAST qPCR Kit (KK4600; KAPA Biosystems) on a LightCycler 96 system (Roche). Primer sequences were as follows: *Zbtb32* sense, 5′-GGTACAGTTAGCGGCTAGACT-3′, and antisense, 5′-GGAAGGGCTTATGTCTTCAACC-3′; *Plk1* sense, 5′-CTTCGCCAAATGCTTCGAGAT-3′, and antisense, 5′-TAGGCTGCGGTGAATTGAGAT-3′; *Cdc20* sense, 5′-CAGCCTGGAGACTACATATCCT-3′, and antisense, 5′-CGGAGTGACTGGTCATGTTTC-3′; *Mcm5* sense, 5′-CAGAGGCGATTCAAGGAGTTC-3′, and antisense, 5′-CGATCCAGTATTCACCCAGGT-3′; *Gapdh* sense, 5′-TCCCACTCTTCCACCTTCGA-3′, and antisense, 5′-AGTTGGGATAGGGCCTCTCTT-3′; *Ccnd2* sense, 5′-TGAAT

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TACCTGGACCGTTTCTTG-3′, and antisense, 5′-AGAGTTGTCGGTGT AAATGCAC-3′, and *E2f1* sense, 5′-TGCAGAAACGGCGCATCTAT-3′, and antisense, 5′-CCGCTTACCAATCCCCACC-3′.

For quantification of the difference in expression of key genes in MBC subsets relative to their expression in naive B cells, the change in cycling threshold (ΔΔCt) was calculated for each gene of interest (GOI) according to the following formula: ΔCt(GOIsubset – GAPDHsubset) – ΔCt(GOINaive – GAPDHNaive), where 'GAPDH' indicates expression of the gene encoding glyceraldehyde phosphate dehydrogenase. Results were calculated as relative change ($2^{-\Delta\Delta Ct}$) for each gene.

Statistics. The Mann-Whitney test or the *t*-test were used for statistical analyses; all comparisons were two-tailed. Results were analyzed with Prism software (GraphPad) and significance was determined at the 95% confidence level. All bar graphs use the mean as a center value. No specific randomization or blinding protocol was used.

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