

# Distinction of the memory B cell response to cognate antigen versus bystander inflammatory signals

Micah J. Benson, Raul Elgueta, William Schpero, Michael Molloy, Weijun Zhang, Edward Usherwood, and Randolph J. Noelle

Department of Microbiology and Immunology, Dartmouth Medical School and the Norris Cotton Cancer Center, Lebanon, NH 03756

**The hypothesis that bystander inflammatory signals promote memory B cell ( $B_{MEM}$ ) self-renewal and differentiation in an antigen-independent manner is critically evaluated herein. To comprehensively address this hypothesis, a detailed analysis is presented examining the response profiles of B-2 lineage  $B220^+IgG^+$   $B_{MEM}$  toward cognate protein antigen in comparison to bystander inflammatory signals. After *in vivo* antigen encounter, quiescent  $B_{MEM}$  clonally expand. Surprisingly, proliferating  $B_{MEM}$  do not acquire germinal center (GC) B cell markers before generating daughter  $B_{MEM}$  and differentiating into plasma cells or form structurally identifiable GCs. In striking contrast to cognate antigen, inflammatory stimuli, including Toll-like receptor agonists or bystander T cell activation, fail to induce even low levels of  $B_{MEM}$  proliferation or differentiation *in vivo*. Under the extreme conditions of adjuvanted protein vaccination or acute viral infection, no detectable bystander proliferation or differentiation of  $B_{MEM}$  occurred. The absence of a  $B_{MEM}$  response to non-specific inflammatory signals clearly shows that  $B_{MEM}$  proliferation and differentiation is a process tightly controlled by the availability of cognate antigen.**

## CORRESPONDENCE

Randolph J. Noelle:  
Randolph.Noelle@  
Dartmouth.edu  
OR  
Micah J. Benson:  
Benson@IDI.Harvard.edu

Abbreviations used: ASC, antibody-secreting cell; BCR, B cell receptor;  $B_{MEM}$ , memory B cell; GC, germinal center; IF, immunofluorescence; KLH, keyhole limpet hemocyanin; MZ, marginal zone; NF, naive follicular; NP, 4-hydroxy-3-nitrophenylacetyl; PB, plasma-blast; PC, plasma cell; PE, phycoerythrin; SHM, somatically hypermutated; sPE, soluble PE; TLR, Toll-like receptor; VACV<sub>WR</sub>, vaccinia virus Western reserve strain.

Life-long immunity is central to the survival of the host. This immunity is engendered by the persistence of memory T cells, memory B cells ( $B_{MEM}$ ), and long-lived BM plasma cells (PCs; Amanna et al., 2007; Dörner and Radbruch, 2007). Long-lasting B cell-mediated immunity has been referred to as serological memory (Traggiai et al., 2003) and can be sustained by recurrent antigen exposure. In the absence of periodic exposure to antigen, it is thought that the production of inflammatory signals to unrelated antigens serve as mediators sustaining serologic memory through activation of  $B_{MEM}$  in a noncognate manner. The *in vitro* and *in vivo* response profiles of antigen-specific  $B_{MEM}$  to cognate antigen and “nonspecific” or bystander inflammatory mediators are analyzed herein to critically evaluate the mechanisms underlying the maintenance of serological memory.

$B_{MEM}$  and long-lived PCs are the products of the germinal center (GC) reaction and express somatically hypermutated (SHM) immunoglobulin receptors of the switched isotypes (MacLennan, 1994; McHeyzer-Williams and Ahmed, 1999). After  $B_{MEM}$  exit from the GC

reaction, they colonize the splenic marginal zones (MZs) and the B cell follicles, where they reside in a quiescent state for months or, likely, years (Liu et al., 1988, 1991; Schittek and Rajewsky, 1990; Anderson et al., 2007). Upon rechallenge with cognate antigen, histological studies in rats observed that  $B_{MEM}$  egress toward the T cell-rich periarteriolar lymphocytic sheath and, upon receiving T cell help, proliferate and differentiate into plasmablasts (PBs) situated in both the periarteriolar lymphocytic sheath and in the splenic red pulp (Liu et al., 1991). Kinetic studies indicate that the generation of this response is rapid and peaks  $\sim$ 4–5 d after secondary antigen encounter (Liu et al., 1991; McHeyzer-Williams et al., 2000; Minges Wols et al., 2007). The PB response dissipates shortly after its initiation, with a subset of PBs further differentiating into long-lived PCs resident in the BM (Liu et al., 1991; Manz et al.,

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1997; McHeyzer-Williams et al., 2006). Shortly after recall,  $B_{MEM}$  cells are once again present in their initial anatomical positions (Liu et al., 1991). Despite numerous studies carefully mapping out the events of the primary humoral immune response toward protein antigens (Jacob et al., 1991; MacLennan, 1994; Allen et al., 2007), relatively little is known about the events occurring during a secondary humoral immune response, including whether antigen reencounter results in the expression of GC markers by antigen-responding  $B_{MEM}$  and/or if  $B_{MEM}$  form GC structures en route to differentiating into PCs (Liu et al., 1991; MacLennan, 1994; McHeyzer-Williams et al., 2006). As the fate and behavior of antigen-activated  $B_{MEM}$  is critical to our understanding of  $B_{MEM}$  self-renewal, included in this study is a detailed analysis of the *in vivo* response of  $B_{MEM}$  toward cognate antigen.

The mechanisms mediating the survival of  $B_{MEM}$  are unknown. Although  $B_{MEM}$  require phospholipase C  $\gamma$  signaling through the B cell receptor (BCR) for survival, it is clear that the  $B_{MEM}$  BCR need not be permanently engaged with antigen, as  $B_{MEM}$  do not require the presence of persisting antigen or antigen complexes to survive (Maruyama et al., 2000; Anderson et al., 2006; Hikida et al., 2009). It is apparent that  $B_{MEM}$  occupy a survival niche independent from other B-2 lineage cell subsets, as, unlike all other mature cells of the B-2 lineage, they do not require BAFF and APRIL survival signals (Benson et al., 2008; Scholz et al., 2008). At the level of the organism, it is thought that humoral memory as a whole is sustained by the stem cell-like qualities of  $B_{MEM}$ , with this cell type undergoing low levels of self-renewal and replenishment of the long-lived PC pool over the lifetime of the organism (Traggiai et al., 2003). To sustain serological memory in the absence of cognate antigen, it has been hypothesized that quiescent  $B_{MEM}$  are periodically activated by Toll-like receptor (TLR) agonists or bystander T cell help to undergo self-renewal events and differentiate into antibody-secreting cells (ASCs) such as PBs and PCs in an antigen-independent manner (Bernasconi et al., 2002). A crucial element of this theory was the prediction that inflammatory stimuli associated with adaptive immune responses drive the activation of  $B_{MEM}$  in an antigen-independent (i.e., nonspecific) manner *in vivo* (Bernasconi et al., 2002, 2003; Traggiai et al., 2003). Whether  $B_{MEM}$  are indeed reactive to bystander inflammatory stimuli *in vivo* has not yet been tested from the vantage point of  $B_{MEM}$ .

In this work, we detail the ability of  $B_{MEM}$  to undergo self-renewal and to differentiate into ASCs in response to both antigen-dependent and bystander inflammatory stimuli. In studying these events, we also demonstrate that during clonal expansion in response to cognate antigen,  $B_{MEM}$  do not express a GC B cell phenotype, and that GC structures are not generated en route to differentiating into ASCs. We find that  $B_{MEM}$  are responsive to inflammatory stimuli *in vitro*, with activated  $B_{MEM}$  displaying a markedly enhanced capacity to differentiate into ASCs when compared with naive follicular (NF) B cells. However, *in vivo*, in response to TLR

agonists, polyclonal T cell activation, protein vaccination, and even acute vaccinia virus infection,  $B_{MEM}$  neither clonally expand nor differentiate into ASCs, and thus appear ignorant to overt bystander inflammatory signals.

## RESULTS

### Antigen-specific and polyclonal $B_{MEM}$ cells are responsive to TLR agonists *in vitro* and display an enhanced capacity to differentiate into ASCs when compared with NF B cells

A comprehensive analysis of  $B_{MEM}$  responsiveness to antigen and bystander inflammatory stimuli was undertaken both *in vitro* and *in vivo*. The term “bystander” is defined herein as stimuli acting on  $B_{MEM}$  independent of BCR engagement. In these studies, we tracked  $B_{MEM}$  specific for the thymus-dependent protein antigen R-phycoerythrin (PE), as immunization of BALB/c mice with this fluorophore elicits a detectable population of PE-binding  $B_{MEM}$  at a high resolution (referred to as PE- $B_{MEM}$ ; Hayakawa et al., 1987). After immunization with PE adsorbed to alum i.p., PE- $B_{MEM}$  are readily detectable in the spleen at 0.01–0.05% of total splenocytes beginning 21 d after immunization, with this population still present 60 d after priming (Fig. S1; Hayakawa et al., 1987; Schittek and Rajewsky, 1990; Benson et al., 2008). In nonimmunized mice, a minute naive PE-binding population is observed at a 10-fold lower frequency (Fig. S1). The PE- $B_{MEM}$  in immunized mice are predominantly B220<sup>+</sup>PE<sup>+</sup>CD38<sup>+</sup>IgD<sup>−</sup>IgG1<sup>+</sup>CD80<sup>hi</sup> and express a phenotype consistent with long-lived affinity-matured  $B_{MEM}$  (Fig. S1; Anderson et al., 2007). In all experiments (unless indicated otherwise), PE- $B_{MEM}$  from PE/alum-immunized mice were analyzed between days 60 and 90 after primary immunization.

The response of PE- $B_{MEM}$  to inflammatory stimuli was first analyzed by examining the response profile of  $B_{MEM}$  to a panel of TLR agonists *in vitro*. We also included two other sources of  $B_{MEM}$  in these *in vitro* analyses to first ensure that the results were reproducible in multiple systems and to test whether  $B_{MEM}$  activation occurs in the absence of BCR cross-linking. The second system used  $B_{MEM}$  raised against and specific for the hapten 4-hydroxy-3-nitrophenylacetyl (NP), with this system having been previously characterized (McHeyzer-Williams et al., 1991; Lalor et al., 1992). The third system used  $B_{MEM}$  from a transgenic mouse in which the polyclonal  $B_{MEM}$  population is readily identified. This latter source allowed for the isolation of  $B_{MEM}$  without the need for BCR engagement by antigen, as when PE- $B_{MEM}$  and NP- $B_{MEM}$  are purified using antigen binding to denote specificity, cross-linking the BCR is inevitable.

**PE- $B_{MEM}$  cells.** From PE-immunized mice, PE- $B_{MEM}$  were first enriched by negative selection, and then electronically sorted based on a B220<sup>+</sup>PE<sup>+</sup>dump<sup>−</sup>CD38<sup>+</sup> phenotype, with post-sort analysis indicating high levels of purity (Fig. S2 A).

**NP-specific  $B_{MEM}$  cells.** NP- $B_{MEM}$  were generated in a transgenic mouse strain where Cre recombinase was inserted

in the  $C\gamma 1$  locus, with this strain crossed with the Rosa26-EYFP reporter mouse (Casola et al., 2006). In this mouse, every post-GC B cell expresses YFP, with this strain affording the opportunity to track post-GC B cell subsets by YFP expression (Casola et al., 2006). Immunization of  $C\gamma 1$ -cre/Rosa26-EYFP mice with NP-keyhole limpet hemocyanin (KLH)/alum lead to the generation of NP- $B_{MEM}$  (Fig. S2 B; Casola et al., 2006). After negative enrichment, NP- $B_{MEM}$  were defined as  $B220^+ NIP^+ IgG1/YFP^+ dump^- CD38^+$ , with post-sort analysis indicating purity (Fig. S2 B).

**Polyclonal  $B_{MEM}$  cells.** Polyclonal  $B_{MEM}$  can be clearly enumerated in  $C\gamma 1$ -cre/Rosa26-EYFP mice through expression of YFP, loss of IgD and IgM, and expression of other  $B_{MEM}$  markers. NP-immunized  $C\gamma 1$ -cre/Rosa26-EYFP mice were negatively enriched for  $B_{MEM}$  with polyclonal  $B_{MEM}$  defined as  $B220^+ IgG1/YFP^+ CD38^+ dump^- CD80^+$  and sorted to purity (Fig. 1 C).

**NF B cells.** In parallel, NF B cells were defined as  $B220^+ CD23^+ IgD^+ CD38^+$  and were additionally sorted (Fig. S2 C).

The response of PE- $B_{MEM}$ , NP- $B_{MEM}$ , poly- $B_{MEM}$ , and NF B cells to TLR agonists after 3 d of in vitro culture was analyzed (Fig. 1). PE- $B_{MEM}$  and corresponding NF B cells and IL-4-only controls were stained with CFSE, whereas NP- $B_{MEM}$  and poly- $B_{MEM}$  with NF B cells and IL-4 only controls were stained with DDAO to track proliferation. After culture with the TLR4 agonist LPS, cells were analyzed for surface expression of B220 and CD138. The culture of all NF and  $B_{MEM}$  with IL-4 resulted in a uniform  $B220^+$  population after 3 d (unpublished data). Upon culture of NF B cells and  $B_{MEM}$  with LPS, NF B cells remained primarily  $B220^+ CD138^-$ , whereas all three sources of  $B_{MEM}$  generated a  $B220^{int} CD138^+$  population of cells ( $\sim 21$ –30% of total) representing the phenotype of a ASC (Fig. 1, A and D). The ASCs derived from the three sources of  $B_{MEM}$  were of the IgG isotype and, with the ASCs generated from PE- $B_{MEM}$  and NP- $B_{MEM}$ , specific for either PE or NP, respectively (Fig. 1 G and not depicted). NF B cells generated a high frequency of IgM<sup>+</sup> ASCs (Fig. 1 G). The overall degree of proliferation by the three sources of  $B_{MEM}$  was moderately less than NF B cells, although proliferation did occur, as indicated by comparison to the IL-4-only cultured cells (Fig. 1, B and E). The population of  $B220^{int} CD138^+$  cells generated from poly- $B_{MEM}$  arose in the daughter peaks, showing that  $B_{MEM}$  undergo cell division while differentiating into  $B220^{int} CD138^+$  cells (Fig. 1 F).

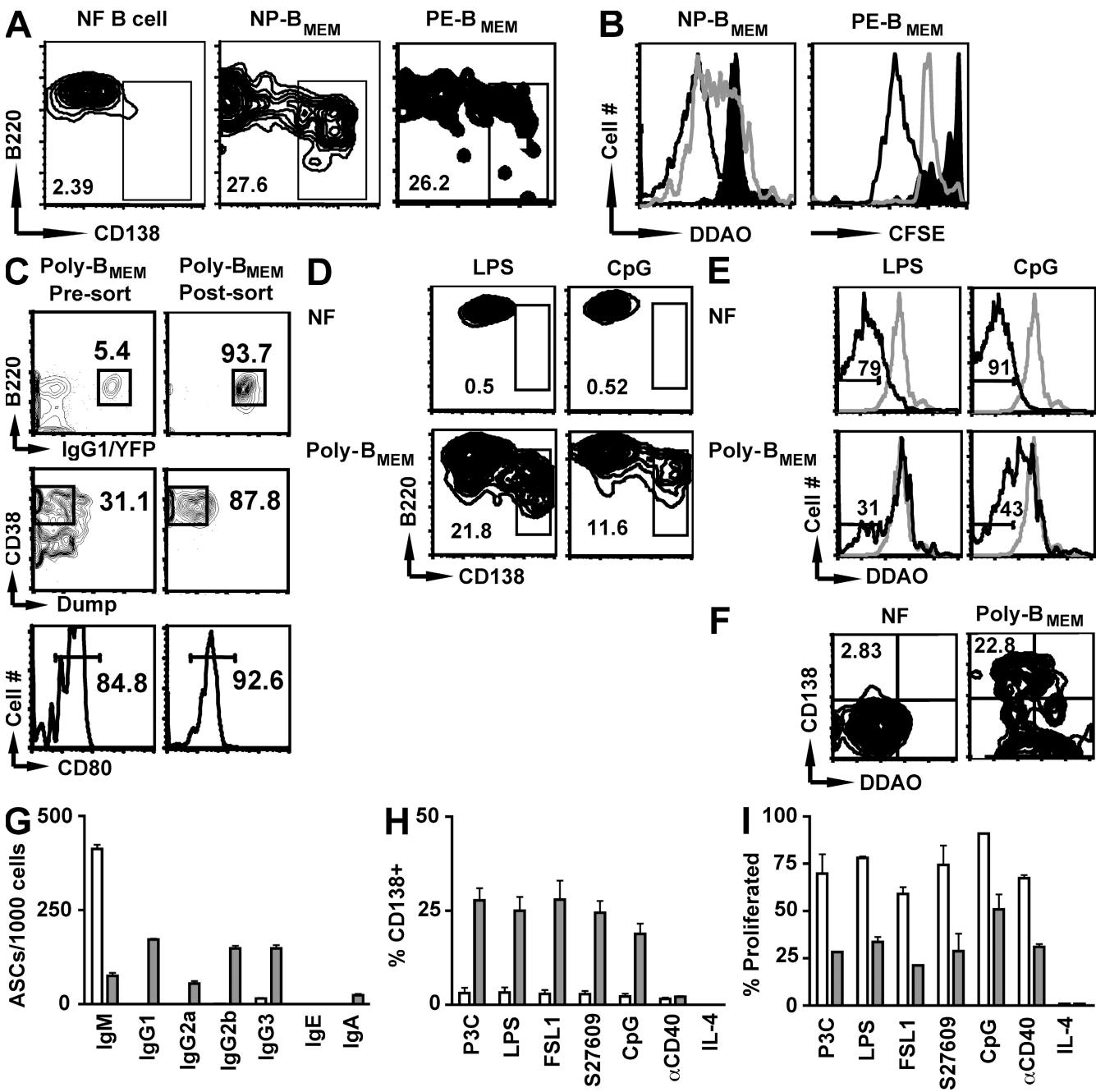
The response of poly- $B_{MEM}$  and NF B cells was analyzed to either Pam3CSK4 (TLR1/2 agonist), heat-killed *Listeria monocytogenes* (TLR2 agonist), poly(I:C) (TLR3 agonist), LPS (TLR4 agonist), flagellin from *Salmonella typhimurium* (TLR5 agonist), FSL-1 (TLR6 agonist), S-27609 (TLR7 agonist), ODN 1826 (TLR9 agonist), and IL-4. Both NF B cells and poly- $B_{MEM}$  were responsive to agonists for TLR1/2, TLR4, TLR6, TLR7, and TLR9 (Fig. 1 I). Both poly- $B_{MEM}$

and NF B responded poorly or did not respond to agonists for TLR2, TLR3, and TLR5 (unpublished data). For each TLR agonist that NF B cells or poly- $B_{MEM}$  responded to,  $B_{MEM}$  generated a sizable population of  $B220^{int} CD138^+$  cells, whereas NF B cells maintained a  $B220^+ CD138^-$  phenotype (Fig. 1 H). NF B cells also proliferated to a greater degree than  $B_{MEM}$  (Fig. 1 I). Finally, it was observed that while engagement of CD40 drove proliferation of both  $B_{MEM}$  and NF B cells,  $B_{MEM}$  did not differentiate into  $B220^{int} CD138^+$  cells (Fig. 1, H–I). Collectively, these data show that  $B_{MEM}$  are sensitive to multiple TLR agonists in vitro and do not require BCR cross-linking to become activated and differentiate into ASCs. Furthermore, upon activation,  $B_{MEM}$  have an enhanced intrinsic capacity to differentiate into CD138<sup>+</sup> ASCs compared with NF B cells. Lastly, CD40 signaling appears to drive  $B_{MEM}$  proliferation, but to block differentiation in vitro.

#### $B_{MEM}$ cells undergo rapid clonal expansion and differentiation into IgG<sup>+</sup> ASCs in response to soluble antigen

Having established that  $B_{MEM}$  are responsive to bystander stimuli in vitro, we used the PE-antigen system to compare  $B_{MEM}$  responsiveness toward cognate antigen versus bystander inflammation in vivo. We first performed a detailed analysis of the secondary humoral immune response to soluble PE (sPE) to define the behavior and kinetics of  $B_{MEM}$  activation in vivo. Unlike naive hosts, it is well-established that previously immunized mice are responsive to low-doses of antigen in the absence of adjuvant (McHeyzer-Williams et al., 2006). This heightened responsiveness results from the combined presence of antigen-reactive  $B_{MEM}$  and CD4<sup>+</sup> memory T cells. In concordance with this, we observed that injection of naive mice with 1  $\mu$ g sPE (or 10  $\mu$ g; not depicted) failed to elicit PE-ASCs (Fig. 2, A and B), whereas PE-immunized mice produced high numbers of IgG<sup>+</sup> PE-ASCs. A robust IgM ASC response was only elicited when naive or PE-immunized mice were injected with 1  $\mu$ g of sPE along with the TLR9 agonist CpG ODN 1826 (CpG; Fig. 2 A). Thus, the IgG<sup>+</sup> PE-specific humoral response generated after sPE injection is likely the direct consequence of  $B_{MEM}$  activation. These data demonstrate that a low level of sPE given in the absence of adjuvant is capable of activating PE- $B_{MEM}$  to differentiate into IgG<sup>+</sup> ASCs.

The in vivo proliferation of PE- $B_{MEM}$  was measured by flow cytometric analysis of BrdU incorporation. PE-immunized mice were injected with sPE, and a 5-d BrdU pulse was performed starting on the day of sPE injection, followed by a chase period. BrdU incorporation by  $B_{MEM}$  was quantified at various times during and after the pulse period. After preenrichment, PE- $B_{MEM}$  were detected by excluding CD4/CD8<sup>+</sup>/IgD/IgM<sup>+</sup> cells via a dump gate and gating them on  $B220^+ PE^+$  cells, with these cells analyzed for BrdU incorporation (Fig. S3 A). PE-immunized mice receiving BrdU, but no antigen, and immunized mice receiving neither antigen nor BrdU were included as negative controls (Fig. 2 C). 2 d after sPE



**Figure 1.** Antigen-specific and polyclonal B<sub>MEM</sub> cells are responsive to TLR agonists in vitro and display an enhanced capacity to differentiate into ASCs compared with NF B cells. NP-B<sub>MEM</sub> and PE-B<sub>MEM</sub> were sorted by FACS, along with NF B cells. NP-B<sub>MEM</sub> and NF B cells were stained with DDAO and PE-B<sub>MEM</sub>, and NF B cells were stained with CFSE. Cells were cultured for 3 d with 10  $\mu$ g/ml LPS, whereupon the cell surface phenotype was analyzed for the expression of B220 and CD138 (A) or DDAO or CFSE (B), with the percentage of B220<sup>+</sup>CD138<sup>+</sup> cells depicted (A, B). Filled histograms, B cells + IL-4; gray lines, B<sub>MEM</sub>; black lines, NF B cells. To analyze the reactivity of B<sub>MEM</sub> to TLR agonists in the absence of BCR cross-linking, poly-B<sub>MEM</sub> were used. Spleens from NP-KLH-immunized C $\gamma$ 1-cre/ROSA-YFP mice were enriched for B<sub>MEM</sub>, with poly-B<sub>MEM</sub> present in the remaining population sorted by the phenotype B220<sup>+</sup>IgG1/YFP<sup>+</sup>CD38<sup>+</sup>Dump<sup>+</sup>CD80<sup>+</sup>. Dump gates, CD43<sup>+</sup>IgD<sup>+</sup>IgM<sup>+</sup> cells (C). Both poly-B<sub>MEM</sub> and NF B cells were labeled with DDAO and cultured for 3 d in the presence of either 10  $\mu$ g/ml LPS or 5  $\mu$ M CpG, whereupon the cell surface phenotype was analyzed for the expression of B220 and CD138 (D) and DDAO dye dilution (E). (E) Gray lines, B cells + IL-4; black lines, NF or poly-B<sub>MEM</sub> as indicated. Proliferation as a function of CD138 expression is shown, with the percentage of cells that have diluted DDAO and are expressing CD138 shown (F). The ability of cells derived from either NF B cells (open bars) or B<sub>MEM</sub> (filled bars) after 3 d of LPS culture to secrete antibody of the IgM, IgG1, IgG2a, IgG2b, IgG3, IgE, and IgA isotypes was analyzed by ELISpot analysis (F). The response profiles of poly-B<sub>MEM</sub> and NF B cells to a panel of TLR agonists was examined after 3 d of culture with the indicated agonist. The agonists used, their abbreviations, the receptors for which they are specific for, and the concentrations used are as follows: Pam3CSK4 (P3C), TLR1/2, 1  $\mu$ g/ml; LPS (LPS), TLR4, 10  $\mu$ g/ml; FSL-1 (FSL1), TLR6/2, 1  $\mu$ g/ml; S-27609 (609), TLR7, 1  $\mu$ g/ml; CpG ODN 1826 (CpG), TLR9, 5  $\mu$ M,  $\alpha$ CD40

rechallenge,  $B_{MEM}$  began to proliferate, with this response peaking by day 4 (Fig. 2 C and Fig. S3 A). In mice analyzed during the BrdU chase period (days 11 and 24 after sPE rechallenge), a proportion of  $B_{MEM}$  remained BrdU<sup>+</sup> at a percentage similar to that observed at the peak of the response (35.5%  $\pm$  SEM 4.6 and 38.4%  $\pm$  SEM 5.1 versus 48.9%  $\pm$  SEM 5.8, respectively), indicating that 71–77% of  $B_{MEM}$  proliferation occurs within the first 5 d after antigen rechallenge (Fig. 2 C and Fig. S3 A). A second PE-binding population was also observed in these experiments, with this population displaying a B220<sup>−</sup>PE<sup>+</sup> phenotype (Fig. S3 A). When the BrdU incorporation frequencies by the B220<sup>−</sup>PE<sup>+</sup> population in PBS versus sPE-immunized mice were compared, no differences were observed between the two groups over the 24-d kinetic analysis (unpublished data). This indicates that the B220<sup>−</sup>PE<sup>+</sup> population does not respond to sPE, and as antigen-responsiveness is a crucial element of adaptive memory, we excluded these cells from further analysis. As BrdU labeling measures the accumulation of proliferation over the time of the BrdU “pulse” period, we also analyzed  $B_{MEM}$  expression of Ki-67 at various times after antigen challenge (Fig. 2 F). Ki-67 expression is an indicator of all stages of the cell cycle and provides a snapshot of the proliferative state of the  $B_{MEM}$  at a given point in time (Gerdes et al., 1984). PE- $B_{MEM}$  are almost uniformly Ki-67<sup>−</sup>, which is indicative of their quiescent state (Fig. 2 F). By days 3 and 4 after sPE rechallenge, 76–77% of  $B_{MEM}$  cells were Ki-67<sup>+</sup>. At day 5, Ki-67 expression began to dissipate, and most PE- $B_{MEM}$  exhibited decreased Ki-67 expression on day 7, with this decrease more pronounced by day 11. By day 24, PE- $B_{MEM}$  were once again uniform in their lack of Ki-67 expression (Fig. 2 F). In correlating the expression pattern of Ki-67 by  $B_{MEM}$  (Fig. 2 F) with the 5-d BrdU pulse followed by a chase period (Fig. 2 C), the results indicate that the majority of  $B_{MEM}$  proliferation occurs between days 2 and 5 after sPE rechallenge. In addition, the presence of BrdU<sup>+</sup>  $B_{MEM}$  24 d after sPE injection provides direct evidence that  $B_{MEM}$  generate daughter  $B_{MEM}$  after secondary antigen encounter.

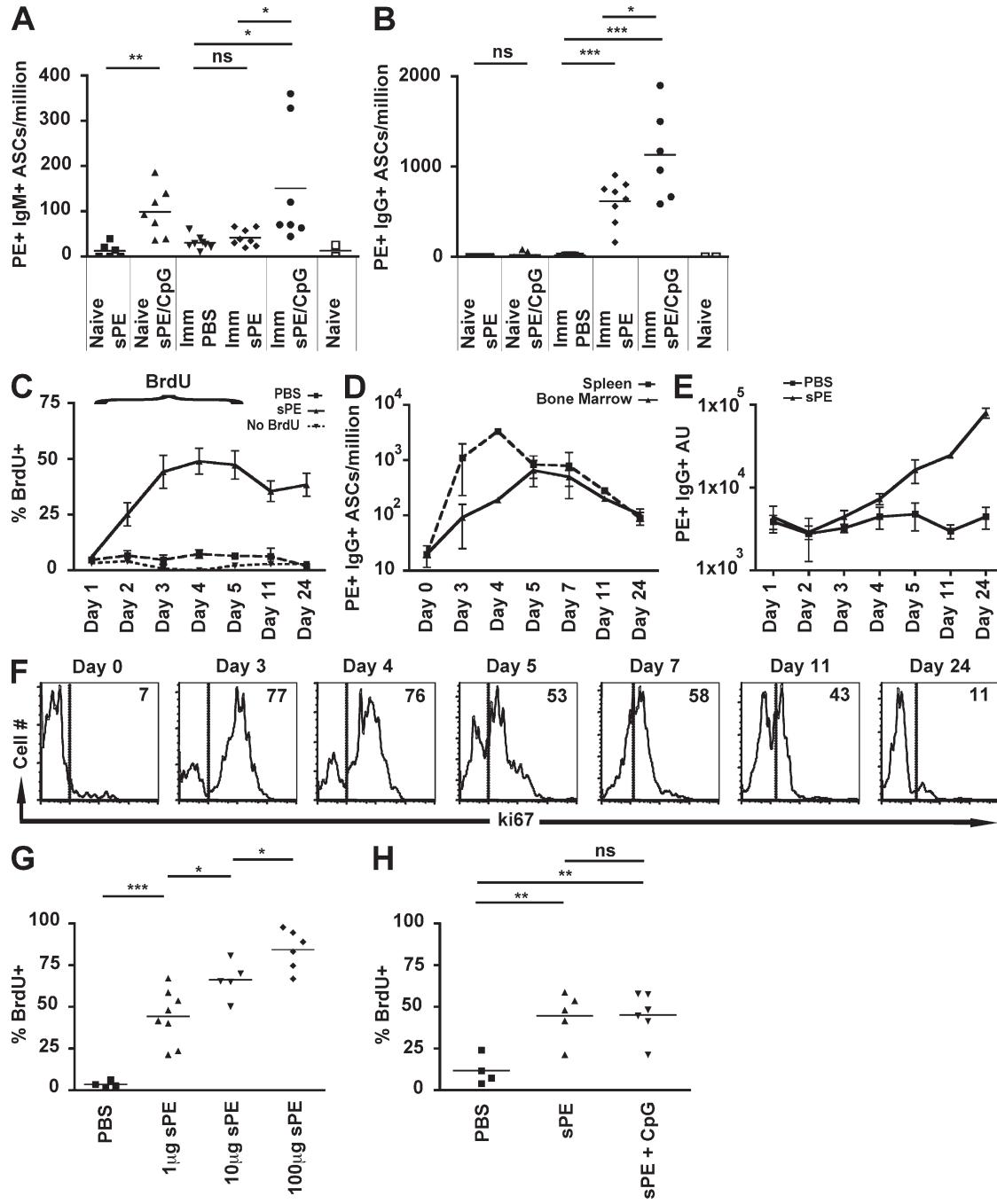
A rapid increase in the numbers of IgG<sup>+</sup> PE-ASCs in both the spleen and BM was observed 3 d after sPE rechallenge (Fig. 2, B and D). This burst in ASC numbers peaked 4 d after challenge in the spleen and 5 d in the BM, after which ASC numbers gradually subsided over the next 20 d (Fig. 2 D). Corresponding to the increase of ASCs after rechallenge was an increase in anti-PE IgG serum titers increasing 10-fold over the next 20 d (Fig. 2 E). These data show that ASCs are rapidly generated within 3 d after antigen rechallenge and are present to substantial degrees in both the spleen and the BM. Soon thereafter, ASC numbers declined, indicating that short-lived ASCs are present in both organs.

The observation that ~50% of  $B_{MEM}$  respond to sPE as indicated by BrdU incorporation (Fig. 2 C and Fig. S3 A) led us to question whether either the antigen dose or the absence of adjuvant during antigen administration was limiting the frequency of responding  $B_{MEM}$ . To test this, we varied the amount of sPE injected or added CpG during antigen rechallenge, with BrdU administration concurrently initiated. When analyzed 3 d later, it was observed that the dose of antigen positively correlated with the percentage of proliferating  $B_{MEM}$  (Fig. 2 G and Fig. S3 B). The addition of CpG had no further impact on the frequency of responding PE- $B_{MEM}$  (Fig. 2 G and Fig. S3 C). However, the addition of CpG to sPE modestly increased the number of splenic IgG<sup>+</sup> PE-ASCs elicited after 3 d when compared with sPE alone (Fig. 2 B). In sum, these data indicate that antigen can be a limiting factor in dictating the frequency of responding  $B_{MEM}$ . Notably, TLR9 agonists failed to enhance the frequency of activated  $B_{MEM}$ . This point indicates that CpGs alone may not play a role in driving *in vivo*  $B_{MEM}$  activation.

#### $B_{MEM}$ cells proliferate upon *in vivo* antigen encounter and do not express GC markers or generate GC structures

The rapid kinetics of the  $B_{MEM}$  response led us to question whether  $B_{MEM}$  form GCs after antigen encounter. Clonally expanding  $B_{MEM}$  were tracked to define their transitional stages en route to differentiating into ASCs. Under steady-state conditions, PE- $B_{MEM}$  displayed a GL7<sup>−</sup>PNA<sup>−</sup>Ki-67<sup>−</sup>CD38<sup>+</sup> phenotype as anticipated (Fig. 3 A; Lalor et al., 1992). As a positive control for GC markers, Peyer's patches were harvested and gated on B220<sup>+</sup>IgD<sup>−</sup> cells, a population known to be primarily GC B cells, with these cells exhibiting a GL7<sup>+</sup>PNA<sup>+</sup>Ki-67<sup>hi</sup>CD38<sup>−</sup> phenotype (Butcher et al., 1982). When PE- $B_{MEM}$  were harvested and analyzed 3 d after sPE challenge, they were GL7<sup>−</sup>PNA<sup>−</sup>Ki-67<sup>hi</sup>CD38<sup>+</sup>, indicating these cells had responded to antigen because of Ki-67 expression, yet failed to express the GC markers GL7 and PNA (Fig. 3 A). It was plausible that the failure of antigen-responding  $B_{MEM}$  to gain a GC phenotype was caused by the solitary use of sPE, and that the induction of a GC phenotype requires the presence of an adjuvant. Therefore, CpG was added to sPE, and 3 d later, the phenotype of the responding  $B_{MEM}$  was analyzed. The addition of CpG to sPE again elicited GL7<sup>−</sup>PNA<sup>−</sup>Ki-67<sup>hi</sup>CD38<sup>+</sup>  $B_{MEM}$  (Fig. 3 A). It was considered possible that  $B_{MEM}$  gain a GC phenotype later than 3 d after antigen encounter, so the phenotype of  $B_{MEM}$  was analyzed at later points in time. The results indicate that PE- $B_{MEM}$  maintain a GL7<sup>−</sup>PNA<sup>−</sup>CD38<sup>+</sup> phenotype throughout the cell cycle (Fig. 3 B). These data define a proliferating state of  $B_{MEM}$  occurring shortly after *in vivo* antigen encounter,

( $\alpha$ CD40), CD40, 10  $\mu$ g/ml; and IL-4, 10 ng/ml. The percentage of B220<sup>int</sup>CD138<sup>+</sup> cells derived from NF B cells (open bars) or  $B_{MEM}$  (filled bars; H) and the percentage of cells proliferating as measured by dye dilution of DDAO (I) are depicted. Data presented is representative (A–G) or pooled (H and I) from three to five independent experiments. Purified  $B_{MEM}$  are pooled from six to eight mice per experiment, with NF B cells isolated from one mouse per experiment. Results in G–I are expressed as the mean  $\pm$  SEM.



**Figure 2.**  $B_{MEM}$  cells undergo rapid self-renewal and differentiation into IgG<sup>+</sup> PCs in response to soluble antigen. Either naive or PE-immunized mice were challenged with PBS, 1  $\mu$ g sPE, or 1  $\mu$ g sPE + 50  $\mu$ g CpG and 3 d later, IgM<sup>+</sup>, and IgG<sup>+</sup> PE-PCs from the spleen were quantified by ELISPOT (A and B). To measure the proliferation of  $B_{MEM}$ , PE-immunized mice were injected with 5  $\mu$ g sPE or PBS with BrdU added to the drinking water and continued for five days as shown (C). A group of immunized mice were included that were not given BrdU or sPE (C). At the indicated day after rechallenge, the percentage of PE- $B_{MEM}$  incorporating BrdU was measured by FACS (C). In parallel, IgG<sup>+</sup> PE-PCs were quantified in sPE- and PBS-injected mice from both the spleen and BM by ELISpot (D). The levels of IgG<sup>+</sup> PE-specific serum antibody titers present on the indicated days after sPE injection were compared with titers present in PBS injected mice by ELISA (E). The expression levels of intracellular Ki-67 by PE- $B_{MEM}$  ( $Dump^{-}B220^{+}PE^{+}$ ; pregates not shown;  $Dump$ ,  $CD4^{+}CD8^{+}IgD^{+}IgM^{+}$ ) was measured at the indicated time points after sPE injection by FACS, with representative plots depicted (F). Plots were stained and analyzed on the same day. To examine the impact of varying sPE dose during rechallenge, PE-immunized mice were rechallenged with 1, 10, 100  $\mu$ g sPE or PBS only, with BrdU given daily in the drinking water. After 3 d, the percentage of PE- $B_{MEM}$  incorporating BrdU was measured by FACS (G). The same experimental setup used in G was used in H, except mice were injected with PBS, 1  $\mu$ g sPE, or 1  $\mu$ g PE with 50  $\mu$ g CpG, with BrdU incorporation by PE- $B_{MEM}$  again depicted. Three to five mice per experimental group were used per experiment. Data presented are representative of two to three independent experiments, with A and B pooled from two independent experiments. Error bars in C–E are expressed as the mean  $\pm$  SEM. AU, arbitrary units.

with these  $B_{MEM}$  expressing a GL7<sup>-</sup>PNA<sup>-</sup>Ki-67<sup>hi</sup>CD38<sup>+</sup> phenotype.

To investigate whether antigen-activated  $B_{MEM}$  generate GCs, GC structures were quantified in spleens where PE- $B_{MEM}$  were either unchallenged or were responding to sPE or sPE + CpG by immunofluorescence (IF) microscopy. As a positive control for the presence of GCs, naive BALB/c were immunized with PE/alum and spleens were examined 10 d later at the height of the GC response. To ensure that PE- $B_{MEM}$  in the sPE- and sPE + CpG-treated groups and the PE-GC B cells in the D10 PE/alum immunized groups were present and proliferating in these mice, FACS analysis was performed on half of the spleens, with BrdU incorporation by B220<sup>+</sup>PE<sup>+</sup> cells analyzed (Fig. S4). Upon IF evaluation, in the D10 PE/alum immunized spleens, GL7<sup>+</sup> clusters were observed and proximal to CD35<sup>+</sup> follicular DCs, with these structures detected in ~50% of the B cell follicles (Fig. 3, C and D). These GL7<sup>+</sup> cell clusters were minimally present in the PBS-, sPE-, and sPE + CpG-treated groups (Fig. 3, C and D). Importantly, there was no significant increase in the number of GCs present in spleens where  $B_{MEM}$  were activated by sPE or sPE + CpG in comparison to PBS controls (Fig. 3 D). These data, coupled with the GL7<sup>-</sup>PNA<sup>-</sup>Ki-67<sup>hi</sup>CD38<sup>+</sup> phenotype displayed by  $B_{MEM}$  after in vivo antigen-dependent activation, demonstrate that GCs are not created by  $B_{MEM}$  after antigen rechallenge.

#### Analysis of the in vivo $B_{MEM}$ cell response to TLR agonists, CD40 agonists, and bystander T cell help

The in vivo  $B_{MEM}$  response to TLR agonists, CD40 agonists, and bystander T cell help was determined. PE-immunized mice were injected with PBS, sPE as a positive control, combinations of TLR agonists, agonistic  $\alpha$ CD40, and/or  $\alpha$ CD3. We treated mice with agonist doses at or greater than doses known to have an optimal biological effect in vivo (Ferran et al., 1990; Ahonen et al., 2004). In data where 50  $\mu$ g of CpG was used, similar data were obtained using 100  $\mu$ g (unpublished data). Furthermore, the efficacy of the TLR agonists was confirmed by observing an induction in the expression of CD86 by both NF B cells and CD11c<sup>+</sup> DCs 24 h after TLR agonist injection in vivo (unpublished data). PE- $B_{MEM}$  proliferation was tracked by BrdU incorporation over a period of 3 d (Fig. S5 A). NF B cells were included in this analysis and were identified as B220<sup>+</sup>IgD<sup>+</sup> cells (Fig. S5 B). Injection with PBS alone elicited very low amounts of BrdU incorporation by both NF B cells and PE- $B_{MEM}$ , whereas injection with sPE elicited only  $B_{MEM}$  proliferation (Figs. 4, A and B, and Fig. S5). It was observed that both  $B_{MEM}$  and NF B cells proliferate in response to injection with agonistic  $\alpha$ CD40 antibody alone or coupled with LPS or CpG, with the mean proliferation of both subsets as measured by BrdU incorporation between 25 and 50% (Fig. 4, A and B, Fig. S5). After in-

jection with LPS, CpG, or  $\alpha$ CD3 alone or  $\alpha$ CD3 and LPS or CpG, no proliferation was observed for either NF B cells or PE- $B_{MEM}$  (Fig. 4, A and B, and Fig. S5). These data indicate that both NF B cells and PE- $B_{MEM}$  proliferate in response to  $\alpha$ CD40 signals in vivo, whereas TLR agonists or activation of T cells with  $\alpha$ CD3 did not induce  $B_{MEM}$  proliferation.

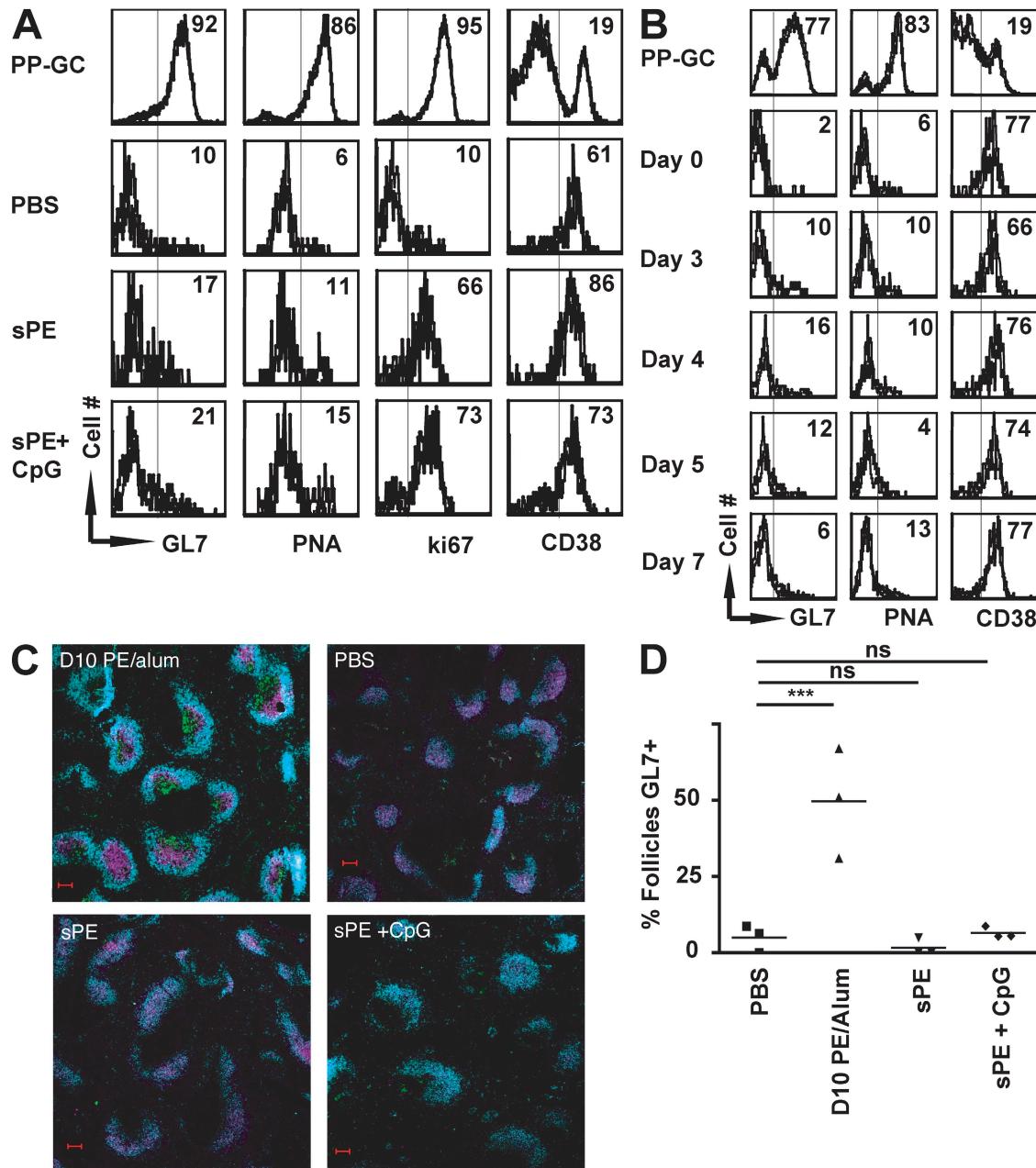
To determine whether  $B_{MEM}$  differentiate into ASCs after signaling through CD40, PE-immunized mice were injected with PBS, sPE, CpG,  $\alpha$ CD40, or both  $\alpha$ CD40 and CpG. IgG<sup>+</sup> PE-ASCs after agonist injection were quantified, and only in the sPE challenged mice was an increase in IgG<sup>+</sup> PE-ASC numbers observed (unpublished data). The numbers of BM IgG<sup>+</sup> PE-ASCs after agonist treatment were also analyzed, with serum harvested on the day of agonist injection and again when analyzed 14 d later. The only treatment group in which an increase in numbers of BM IgG<sup>+</sup> PE-ASCs and serum PE-specific IgG was observed in the sPE-treated cohort (Fig. 4, C and D).

These data indicate that TLR4 and TLR9 agonists and the proinflammatory cytokine storm elicited by agonistic  $\alpha$ CD3 treatment are incapable of activating  $B_{MEM}$  and driving their proliferation or differentiation in vivo. Engagement of CD40 (either alone or coupled with TLR4 and TLR9 agonists) drives both NF B cell and  $B_{MEM}$  proliferation, but is incapable of mediating their differentiation into ASCs.

#### $B_{MEM}$ cells do not proliferate or differentiate into ASCs in vivo after immunization with an irrelevant protein antigen or vaccinia virus infection

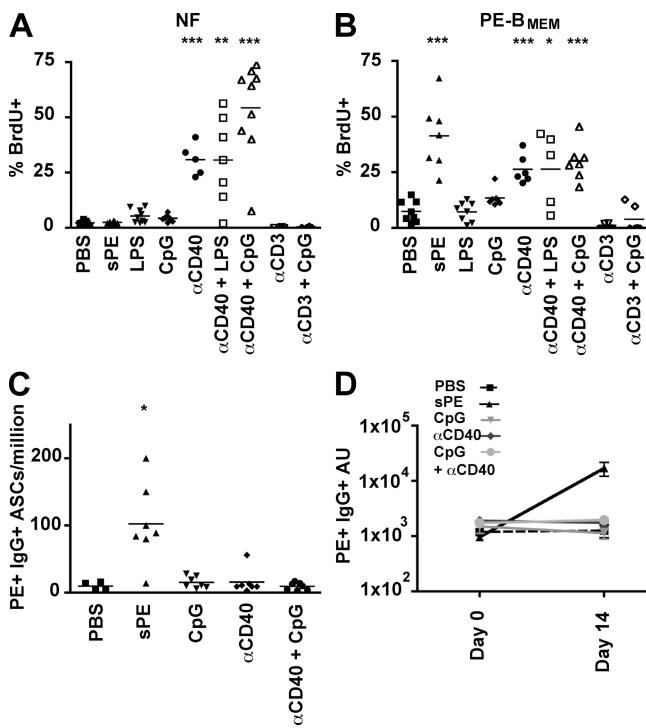
The capacity of PE- $B_{MEM}$  to proliferate and differentiate in response to either immunization with NP-KLH/alum administered with CpG or infection with vaccinia virus Western Reserve strain (VACV<sub>WR</sub>) was analyzed. The levels of  $B_{MEM}$  proliferation and their differentiation into ASCs were measured 17 d later with this time chosen to allow for the full development of an adaptive immune response against either NP-KLH or VACV<sub>WR</sub>. After immunization with VACV<sub>WR</sub> or NP-KLH, PE- $B_{MEM}$  did not incorporate any more BrdU (15.9%  $\pm$  SEM 1.8 and 16.6%  $\pm$  SEM 2.3, respectively) and then after injection with PBS alone (19.22%  $\pm$  SEM 1.5; Fig. S6 C and Fig. 5 A). As anticipated, sPE induced robust PE- $B_{MEM}$  cell proliferation, with the majority (73%  $\pm$  SEM 2.8) of cells BrdU<sup>+</sup> (Fig. S6 C and Fig. 5 A). The generation of IgG<sup>+</sup> PE-ASCs was also measured by comparing the levels of PE<sup>+</sup> IgG<sup>+</sup> serum antibody titers present before and after immunization and by quantifying the number of IgG<sup>+</sup> PE-ASCs present in the spleen and BM by ELISPOT. VACV<sub>WR</sub> and NP-KLH immunizations did not impact the number of IgG<sup>+</sup> PE-ASCs present in either the spleen or the BM 17 d after immunization (Fig. 5, B and C). There was also no difference in the levels of IgG<sup>+</sup> PE-specific serum antibody titers after VACV<sub>WR</sub> or NP-KLH immunization (Fig. 5 D). Consistent

Each point in A, B, G, and H represents an individual mouse, with horizontal line representing the mean. Results of a Student's *t* test are shown as comparisons between the indicated experimental groups: ns, no significance; \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.



**Figure 3.**  $B_{MEM}$  cells proliferate upon in vivo antigen encounter and do not express GC markers or generate GC structures. PE-immunized mice were injected with PBS, 10  $\mu$ g sPE, or 10  $\mu$ g sPE + 50  $\mu$ g CpG. 3 d later, PE- $B_{MEM}$  (Dump $\cdot$ B220 $^+$ PE $^+$ ; pregates not shown and Dump = CD4 $^+$ CD8 $^+$ IgD $^+$ IgM $^+$ ) in each treatment group were analyzed for their expression of GL7, PNA, Ki-67, and CD38 by FACS. Included in this analysis were lymphocytes harvested from the Peyer's patches of the same mice and gated on B220 $^+$ IgD $^-$  (PP-GC; pregates not depicted). Representative data depicted are histograms of indicated phenotypic marker (A). A similar experiment was performed with responding PE- $B_{MEM}$  examined at various time points after 10  $\mu$ g sPE rechallenge (B). To detect the presence of GCs in mice harboring antigen-responding  $B_{MEM}$ , PE-immunized mice were rechallenged with PBS, sPE, or sPE + CpG. Included in the analysis were BALB/c mice that had received a primary PE/alum immunization 10 d prior. GCs in each group were quantified by IF microscopy (C) and are represented as the percentage of splenic IgD $^+$  B cell follicles harboring GL7 $^+$  foci, with 50–75 follicles counted/spleen (D). A and B are representative of three experiments, with three to four mice analyzed on each day. The IF experiment contained three mice analyzed from two independent experiments (C and D). For IF images, green represents GL7, blue represents IgD, and purple represents CD35. Bar, 100  $\mu$ m. In D, each point represents an independent mouse, with line representing the mean. Results of an unpaired Student's *t* test in D are shown as a comparison between indicated groups. \*\*\*,  $P < 0.001$ .

with our earlier observations (Fig. 2, D and E), sPE induced a substantial and significant increase in the number of IgG<sup>+</sup> PE-ASCs in both the spleen and BM (Fig. 5, B and C) and led to an ~30-fold increase in the levels of IgG<sup>+</sup> PE-specific serum antibody titers (Fig. 5 D). Both cellular and humoral anti-VACV<sub>WR</sub> immune responses were verified to have occurred by testing for the presence of IFN- $\gamma$ <sup>+</sup> VACV<sub>WR</sub>-specific CD4 and CD8 T cells in spleens harvested from VACV<sub>WR</sub>-infected mice, but not naive mice, and by observing for the presence of anti-VACV<sub>WR</sub> IgG serum antibodies only in infected mice (Fig. S6, A and B).

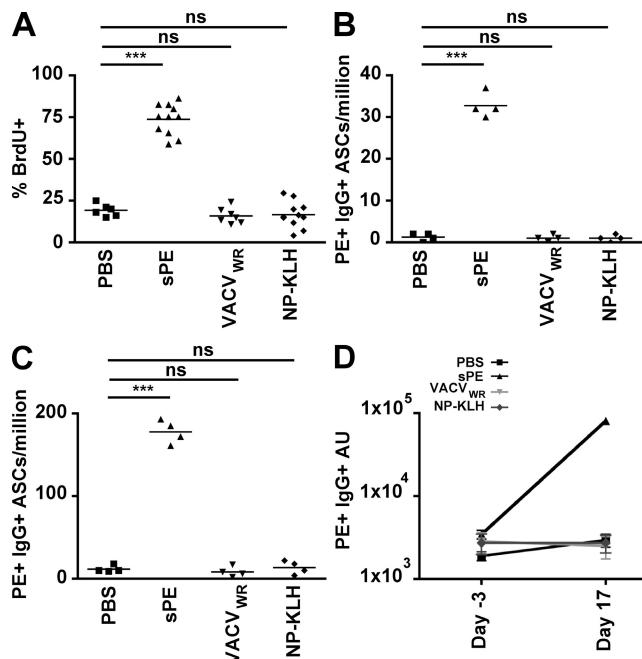


**Figure 4. Analysis of the in vivo B<sub>MEM</sub> cell response to TLR agonists, CD40 agonists, and bystander T cell help.** PE-immunized mice were injected i.p. with PBS, 10  $\mu$ g sPE, 50  $\mu$ g CpG, 50  $\mu$ g LPS, 100  $\mu$ g  $\alpha$ CD40, or 5  $\mu$ g  $\alpha$ CD3 either alone or in combination as indicated. For A and B, BrdU was added to the drinking water at the time of injection and for the duration of the experiment. The percentage of splenic NF B cells and PE-B<sub>MEM</sub> incorporating BrdU was analyzed 3 d later by FACS (A and B, as indicated). To analyze whether CpG and  $\alpha$ CD40 either alone or in combination enhanced the number of PE-PCs in the BM, mice received the indicated agonist and were sacrificed 14 d later, with the number of IgG<sup>+</sup> PE-PCs in the BM quantified by ELISpot (C). Furthermore, serum was harvested on the same day as agonist injection (Day 0), as well as on the day of sacrifice (Day 14), with the titers of IgG<sup>+</sup> PE-specific antibody titers analyzed on both days by ELISA (D). In A–C, three to four mice per experimental group were used per experiment. Data shown is pooled from three independent experiments. In A–C, each dot depicts an individual mouse, with mean indicated by horizontal line. In D, each group contains seven mice, with data expressed as the mean  $\pm$  SEM. AU, arbitrary units. D is the pooled data of two independent experiments. Results of an unpaired Student's *t* test are shown as comparisons between the indicated experimental group and the PBS control group: no denotation, no significance; \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.

These data collectively demonstrate that immunization with NP-KLH and VACV<sub>WR</sub> infection does not induce PE-B<sub>MEM</sub> proliferation or differentiation into ASCs. Thus, B<sub>MEM</sub> are unresponsive to stimuli generated during a bystander immune response.

## DISCUSSION

A detailed analysis of the in vivo secondary immune response of B<sub>MEM</sub> in response to cognate antigen versus noncognate inflammatory bystander signals is presented. These data show that B<sub>MEM</sub> respond robustly upon rechallenge with cognate antigen, but do not acquire the expression of GC markers or form GCs in response to cognate antigen. In striking contrast, the response of B<sub>MEM</sub> to a battery of proinflammatory signals is remarkably silent. It has been proposed that serological



**Figure 5. B<sub>MEM</sub> cells do not proliferate or differentiate into PCs in vivo after immunization with an irrelevant protein antigen or vaccinia virus infection.** PE-immunized mice were injected i.p. with PBS, 10  $\mu$ g sPE, 2  $\times$  10<sup>6</sup> PFU VACV<sub>WR</sub>, or 100  $\mu$ g NP-KLH/alum + 50  $\mu$ g CpG, as indicated. BrdU was added to the drinking water at the time of injection and for the duration of the experiment. After 17 d, the percentage of PE-B<sub>MEM</sub>-incorporating BrdU was quantified by FACS analysis (A). On the same day, the numbers of IgG<sup>+</sup> PE-PCs were quantified in both the spleen and BM for each treatment group by ELISpot (B and C, respectively). Serum was harvested 3 d before immunization and again on day 17, with IgG<sup>+</sup> PE-specific serum antibody titers from both time points shown (D). Horizontal lines represent the mean (A–C). In A–C, three to four mice per experimental group were used per experiment. Data shown in 5A is pooled from two independent experiments, and data shown in B and C is representative of one of these experiments. Results in D are expressed as the mean  $\pm$  SEM. AU, arbitrary units. Seven to eight mice were used in each treatment group. D is the pooled data of two independent experiments. Results of an unpaired Student's *t* test are shown as comparisons between the indicated experimental groups in A–C. \*\*\*, P < 0.001.

memory is sustained by  $B_{MEM}$  responding to bystander inflammatory signals by undergoing proliferation and differentiation. However, the findings of this study indicate that  $B_{MEM}$  ignore these signals (TLR agonists, polyclonal T cell activation, aggressive vaccination, and acute viral infection), as they fail to drive  $B_{MEM}$  proliferation or differentiation *in vivo*.

Despite the preponderance of studies detailing the B cell dynamics of the primary humoral immune response, surprisingly few studies have detailed the events of the secondary humoral immune response. We have tracked  $B_{MEM}$  and demonstrate that cognate antigen drives the rapid clonal expansion of  $B_{MEM}$  and their terminal differentiation into ASCs, with these events occurring in the absence of GC formation. As has been widely reported,  $B_{MEM}$  rapidly differentiate to ASCs upon secondary antigen encounter (Höfer et al., 2006; Minges Wols et al., 2007), with this ASC response first detectable 3 d after antigen challenge in both the spleen and the BM and with the splenic ASC response an order of magnitude greater than that observed in the BM. This wave of ASCs dissipates after 11 d, and it is probable that the remaining ASCs in the BM 24 d after antigen challenge have been culled into the long-lived PC pool (Manz et al., 1997; Höfer et al., 2006; Minges Wols et al., 2007). These data detail the activation of  $B_{MEM}$  and define their proliferation as occurring between days 2 and 5 after antigen encounter. Proliferating  $B_{MEM}$  occupy a transitional state defined as antigen-binding  $B220^+CD38^+Ki-67^+GL7^-PNA^-$  before generating quiescent daughter  $B_{MEM}$  and/or differentiating into ASCs. This blasting state is unique because of the presence of high levels of CD38 expression by proliferating cells; CD38 is a phenotypic marker previously used to denote quiescent NF B cells and  $B_{MEM}$  and is lost upon B cell activation and entry into the GC (Oliver et al., 1997). Our finding that  $B_{MEM}$  cells do not form or enter a GC reaction during their differentiation and expansion is consistent with classical studies indicating that  $B_{MEM}$  might not undergo further SHM and isotype switching upon rechallenge with antigen (Askonas et al., 1970; Okumura et al., 1976; Siekevitz et al., 1987). In these studies, limiting numbers of hapten-specific “parental”  $B_{MEM}$  were adoptively transferred together with carrier-primed T cells into lightly irradiated hosts, followed by immunization of the hosts with soluble hapten-carrier antigen. Upon analyzing the isotype distribution and SHM exhibited by the parental  $B_{MEM}$  and their progeny generated in response to cognate antigen, no change in diversity in either measure was observed. Our data showing that  $B_{MEM}$  neither gain GC markers nor seed GCs are in concordance with these studies. It is apparent that  $B_{MEM}$ , at least in these studies and in our presented data, are not instructed to enhance the affinity or isotype distribution of their antigen receptors during secondary antigen encounter.

Our data indicate that quiescent IgG<sup>+</sup>  $B_{MEM}$  are not activated by TLR agonists or bystander T cell help, and thus do not undergo self-renewal events or differentiate into ASCs in an antigen-independent manner. We should note that our analysis was limited to IgG<sup>+</sup> PE- $B_{MEM}$  and that our experi-

mental system excluded testing whether IgM<sup>+</sup>  $B_{MEM}$  are responsive to bystander inflammatory signals. There is a growing body of evidence both in favor of and in conflict with the hypothesis that serological memory is sustained by the activation of  $B_{MEM}$  by bystander inflammation. Evidence in favor of this theory includes *in vitro* observations in which human  $B_{MEM}$  were shown to have increased sensitivity and to selectively proliferate and differentiate into ASCs in response to bystander stimuli as compared with NF B cells (Bernasconi et al., 2002, 2003). *In vivo* studies in humans found that for single-pathogen specificities, serum antibody levels linearly correlated with the frequency of  $B_{MEM}$  present in a host, indicating that the ASC compartment was genealogically linked to and undergoing replenishment by the  $B_{MEM}$  compartment (Bernasconi et al., 2002). Mouse studies also supported this link between the  $B_{MEM}$  and ASC compartments as elimination of virus-specific  $B_{MEM}$  led to a slow decay of virus-specific ASC numbers over time in comparison to mice still containing  $B_{MEM}$  (Slifka et al., 1998). Lastly, it was observed that during an immune response against a single pathogen, an increase in both blood ASC numbers and serum antibody levels occurred for specificities to other pathogens (Bernasconi et al., 2002). This result was interpreted as evidence that host  $B_{MEM}$  were recipients of antigen-independent stimuli and responded by differentiating into ASCs.

Despite these data suggesting that  $B_{MEM}$  respond to bystander inflammation, more recent studies have put this hypothesis under scrutiny. In particular, further attempts to correlate antigen-specific  $B_{MEM}$  numbers with circulating ASCs in human subjects have been unsuccessful (Amanna et al., 2007). Mouse studies where long-lived ASC numbers were quantified after  $B_{MEM}$  depletion observed no decay in ASC numbers over time, contrary to initial reports (Ahuja et al., 2008; DiLillo et al., 2008). Lastly, a series of studies reported that in mice that were administered a TLR agonist or in humans that were given a vaccine, ASC numbers and serum antibody levels against specificities other than the immunizing antigen did not increase (Di Genova et al., 2006; Amanna et al., 2007; Xiang et al., 2007; Richard et al., 2008).

Our studies show that the *in vitro* sensitivities of  $B_{MEM}$  to activation are not representative of their sensitivities *in vivo* and cast doubt on the hypothesis that  $B_{MEM}$  can be activated by bystander inflammation. Our *in vitro* studies examining the response profiles of murine  $B_{MEM}$  to both antigen-dependent and -independent stimuli extended prior studies using human (Arpin et al., 1997; Bernasconi et al., 2002) and murine (Richard et al., 2008)  $B_{MEM}$ , as we observe that murine  $B_{MEM}$  are sensitive to TLR agonists and have an enhanced intrinsic ability to differentiate into ASCs upon activation with TLR agonists, regardless of whether the BCR is engaged by antigen. Given these data, and our knowledge of the robust response of  $B_{MEM}$  to cognate antigen *in vivo*, an exploration of the response of  $B_{MEM}$  to several nonspecific inflammatory signals *in vivo* demonstrated that  $B_{MEM}$  are in fact unresponsive to these stimuli. The first panel of signals tested was derived from activated T cells and included CD154

expression by these cells, as well as proinflammatory cytokines either alone or together with TLR agonists. We first analyzed the impact of agonistic  $\alpha$ CD40 antibodies on  $B_{MEM}$  proliferation and differentiation. The injection of mice with  $\alpha$ CD40 alone or with TLR4 or TLR9 agonists elicited substantial levels of  $B_{MEM}$  and NF B cell proliferation. Despite this proliferation, these  $B_{MEM}$  did not differentiate into ASCs. These data are in agreement with our in vitro observations, where  $\alpha$ CD40 induced the proliferation of, yet failed to differentiate, poly- $B_{MEM}$  into ASCs. These studies also extend previous in vitro studies where CD40 signaling triggered human  $B_{MEM}$  proliferation, but impeded their differentiation into ASCs (Arpin et al., 1995). Using a second approach, we activated T cells in vivo through the injection of agonistic  $\alpha$ CD3 antibodies, with this treatment given either alone or with TLR agonists. Injection of this antibody is known to elicit rapid and transient activation of T cells, which release a proinflammatory cytokine storm that includes IL-2, IFN- $\gamma$ , and TNF (Ferran et al., 1990; Scott et al., 1990). These stimuli did not induce either  $B_{MEM}$  proliferation or differentiation.

It is highly likely that the in vivo response of  $B_{MEM}$  to overt antibody-mediated CD40 signaling does not represent the hypothetical situation where  $B_{MEM}$  and activated T cells transiently engage in a CD40/CD40L synapse independent of an antigen bridge. As we found no  $B_{MEM}$  proliferation upon vaccination with an irrelevant thymus-dependent antigen or infection with vaccinia virus (a situation generating large numbers of activated CD4 T cells expression CD40L), it is probable that this interaction does not occur and is thus not a mechanism for propagating  $B_{MEM}$  renewal. Instead, this data should be interpreted as in vivo proof that CD40 signaling drives  $B_{MEM}$  proliferation but blocks their differentiation into ASCs, indicating that differentiation occurs upon cessation of CD40 signaling.

Immunization with NP-KLH, along with alum and CpG, and infection with vaccinia virus results in the acute induction of both innate and adaptive immunity.  $B_{MEM}$  ignored the stimuli associated with these responses and neither proliferated nor differentiated into ASCs during the multi-week period in which these immune responses were occurring. In summary, inflammatory signals associated with TLR signaling, T cell activation, and adaptive immune responses are incapable of driving  $B_{MEM}$  proliferation or differentiation into ASCs.

The possibility remains that  $B_{MEM}$  and long-lived PCs might survive for the lifetime of the host, with these populations not requiring self-renewal or replenishment and with serological memory not requiring maintenance. However, this hypothesis requires that  $B_{MEM}$  and PCs survive for perpetuity, and this quality appears unrealistic. From our presented work, we show that  $B_{MEM}$  do not clonally expand or differentiate in response to bystander inflammatory signals. It is possible that there are other signals capable of inducing in vivo  $B_{MEM}$  expansion and differentiation that are not included in this analysis. Alternatively, the maintenance of serological memory may occur via a stochastic rather than an induced

process, with  $B_{MEM}$  not requiring an extrinsic signal to maintain serological memory such as those tested herein.

## MATERIALS AND METHODS

**Mice and immunizations.** These studies were approved by the Institutional Animal Care and Use Committee of Dartmouth College (Lebanon, NH). BALB/c mice were purchased from the National Cancer Institute. RAG<sup>-/-</sup> mice were purchased from The Jackson Laboratory. C $\gamma$ 1-cre mice crossed with Rosa26-EYFP mice have been previously described and were provided by K. Rajewsky (Harvard Medical School, Boston MA) and S. Casola (Fondazione Italiana per la Ricerca sul Cancro Institute of Molecular Oncology Foundation, Milan, Italy; Casola et al., 2006). All animals were maintained in a pathogen-free facility at Dartmouth Medical School. For primary immunizations, 10  $\mu$ g of R-PE (Cyanotech) or 100  $\mu$ g of NP<sub>28</sub>-KLH (Biogenesis Technologies) adsorbed to prepared alum was injected i.p. in a volume of 200  $\mu$ l. For secondary challenge, 10  $\mu$ g of PE (unless indicated otherwise) in PBS in a volume of 200  $\mu$ l was injected i.p. For VACV<sub>WR</sub> infections, virus was propagated and titrated on the 143B cell line as previously described (Fuse et al., 2008), with 2  $\times$  10<sup>6</sup> PFU injected i.p. For BrdU treatment, 0.8 mg/ml was added to the drinking water and changed daily except in Fig. 5 where BrdU was changed every other day.

**Cell preparation.** Single-cell suspensions of lymphocytes were prepared as previously described (Benson et al., 2008). For enrichment of splenocytes for  $B_{MEM}$  before phenotyping and BrdU analysis, CD4<sup>+</sup>, CD8<sup>+</sup>, IgD<sup>+</sup>, and IgM<sup>+</sup> cells were removed by negative depletion using a mix of biotinylated antibodies. Cells were removed using the EasySep Biotin Selection Kit for Mouse Cells (StemCell Technologies). For enrichment of  $B_{MEM}$  before cell sorting, IgD<sup>+</sup> and IgM<sup>+</sup> cells were first removed, and then CD43<sup>+</sup> cells were removed in a two-step process using the EasySep Mouse B Cell Enrichment Kit.

**Cell culture/reagents.** B cells were cultured in RPMI media supplemented with 10% FBS (Atlanta Biologicals), Hepes, 50  $\mu$ M  $\beta$ -ME, and penicillin/streptomycin/L-Glutamine. Sorted B cells were cultured in 96-well round-bottomed plates with 10,000–15,000 cells/well and were cultured at 37°C. For in vitro VACV<sub>WR</sub> CD4 and CD8 recall, BALB/c splenocytes were infected for 2 h with a 10:1 ratio of virus to cells. Infected cells were then plated in a round-bottom 96-well plate with 5  $\times$  10<sup>6</sup> splenocytes harvested from either D17 VACV<sub>WR</sub>-infected or noninfected mice added and cultured for 6 h in the presence of 10  $\mu$ g brefeldin-A and 10 U/ml IL-2. Intracellular staining of IFN- $\gamma$  was performed as previously described (Fuse et al., 2008). The following reagents and vendors were used in this study: CpG-ODN1826 (5'-TCCATGACGTTCTGACGTT-3') with phosphorothioate bases (Eurogentec S.A.), LPS 055:B5, BrdU (Sigma-Aldrich), Pam4CSK4, HKLM, poly(I:C), ST-FLA, and FSL1 (Invivogen), S-27609 (3M Pharmaceuticals), IL-4 (Peprotech),  $\alpha$ CD40 clone FGK45, and  $\alpha$ CD3 clone 145 2C11 antibodies (Klaus Lube; BioExpress).

**Flow cytometry.** Antibodies against the following antigens were used: B220 (clone 6B2), IgG1 (clone A85-1), IgD clone (11-26c), IgM (clone 11-41), CD4 (clone GK1.5), CD8 (clone 2.43), IFN- $\gamma$  (clone XMG1.2), CD43 (clone 1B11), CD38 (clone 90), CD23 (clone B3B4), CD80 (clone 1610-A1), Ki-67 (clone B56), CD138 (clone 281-2), BrdU (clone PRB-1), CD35 (clone 8C12), GL7, and PNA. NIP-PE and PE-binding cells were detected by staining with NIP<sub>20</sub>-PE (generated in-house) or with 2  $\mu$ g/ml PE. For CFSE or DDAO dye dilution, cells were labeled with 5  $\mu$ M CFSE or 10  $\mu$ M DDAO in RPMI at 37°C for 10 min (Invitrogen). Intracellular BrdU (BD) and Ki-67 (eBioscience) staining was performed according to the manufacturers recommendations. Flow cytometry was performed on a refurbished FACSCAN running CellQuest software (BD), with data analysis performed using FlowJo (Tree Star, Inc.). Cell sorts were performed on a FACSaria (BD; Flow Cytometry Facility at Dartmouth Medical School).

**ELISpot/ELISA analysis.** For ELISpot analysis, cells were apportioned to PE, NP<sub>3</sub>-BSA, or polyclonal anti-Ig-coated Multiscreen 96-well plates (Millipore) with twofold serial dilutions made before incubation for 7–18 h at 37°C. PCs were detected by HRP-conjugated or biotinylated anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgE, or IgA polyclonal antibodies (SouthernBiotech.), with streptavidin-HRP used in a second step when necessary. ELISpots were developed as previously described (Benson et al., 2008).

For ELISA analysis, plates were coated with either 10 µg/ml PE or 4 × 10<sup>6</sup> PFU of H<sub>2</sub>O<sub>2</sub>-inactivated VACV<sub>WR</sub> (Hammarlund et al., 2008) overnight in PBS, blocked with PBS + 5% FBS, and washed, and then 1:2,000 diluted serum added at serial 1:2 dilutions. Serum from either a PE hyperimmunized mouse or from a VACV<sub>WR</sub> mouse was included on each plate as a reference between plates and between experiments and used to generate a standard curve, with these serum allotted the value 8,000 arbitrary units. Antibody levels were detected with IgG-AP (SouthernBiotech) and developed with 1 mg/ml pNPP (Sigma-Aldrich) in 0.05 sodium carbonate buffer.

**Immunofluorescence microscopy.** Spleens were presoaked in 15% sucrose/PBS for 30 min and snap-frozen in OCT compound (Sakura Finetek) in a dry ice/ethanol slurry and stored at –80°C. Tissues were sectioned onto slides using a cryostat with 8-µm-thick sections. After drying at room temperature, the tissue was fixed in ice-cold 1:1 acetone/methanol mix for 10 min and allowed to dry, after which sections were outlined with an ImmEdge Pen (Vector Laboratories). Sections were blocked with 10% heat-inactivated normal rat serum/PBS for 1 h, washed, and stained with IgD-Alexa Fluor 647, GL7-FITC, and CD35-biotin, followed by staining with SA-PerCP. After washing, slides were mounted with PermaFluor Aqueous Mounting Media (Thermo Fisher Scientific). Samples were analyzed on a LSM 510 META confocal microscope using LSM software (Carl Zeiss, Inc.).

**Online supplemental material.** Fig. S1 shows FACS gating scheme and phenotype of PE-B<sub>MEM</sub>. Fig. S2 displays the FACS profiles and gating schemes used to sort-purify PE-B<sub>MEM</sub>, NP-B<sub>MEM</sub>, and NF B cells. Fig. S3 contains FACS profiles showing BrdU incorporation by PE-B<sub>MEM</sub> and data corresponding to experiments presented in Fig. 2. Fig. S4 contains FACS plots showing BrdU incorporation by PE-B<sub>MEM</sub> and D10 GC B cells, with these data corresponding to experiments presented in Fig. 3. Fig. S5 contains FACS plots showing BrdU incorporation by PE-B<sub>MEM</sub> and NF B cells after in vivo administration of TLR agonists, CD40 agonists, and bystander T cell help. Data correspond to experiments presented in Fig. 4. Fig. S6 depicts the presence of VACV<sub>WR</sub>-specific CD4 and CD8 cells and VACV<sub>WR</sub>-specific serum IgG in infected hosts. FACS plots are also depicted showing levels of BrdU incorporation by PE-B<sub>MEM</sub> after protein vaccination or viral infection, with data corresponding to experiments depicted in Fig. 5. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090667/DC1>.

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