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Blimp-1 Is Required for the Formation of Immunoglobulin Secreting Plasma Cells and Pre-Plasma Memory B Cells

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Summary

Blimp-1 is a transcriptional repressor able to drive the terminal differentiation of B cells into Ig-secreting plasma cells. We have created mice with a B cell-specific deletion of *prdm1*, the gene encoding Blimp-1. B cell development and the number of B cells responding to antigen appear to be normal in these mice. However, in response to either TD or TI antigen, serum Ig, short-lived plasma cells, post-GC plasma cells, and plasma cells in a memory response are virtually absent, demonstrating that Blimp-1 is required for plasmacytic differentiation and Ig secretion. In the absence of Blimp-1, CD79b$^+$B220$^-$ pre-plasma memory B cell development is also defective, providing evidence that this subset is an intermediate in plasma cell development. B cells lacking Blimp-1 cannot secrete Ig or induce μS mRNA when stimulated ex vivo. Furthermore, although *prdm1*$^{-/-}$ B cells fail to induce XBP-1, XBP-1 cannot rescue plasmacytic differentiation without Blimp-1.

Introduction

Plasma cells (PCs), the critical immune effector cells dedicated to secretion of antigen-specific immunoglobulin (Ig), develop at three distinct stages of antigen-driven B cell development (Calame, 2001). Short-lived PCs emerge in response to both thymus-independent (TI) and thymus-dependent (TD) antigens (Ho et al., 1986) in the first week after antigen exposure in extrafollicular foci of secondary lymphoid organs (Jacob et al., 1991a). These PCs have half-lives of 3–5 days (Ho et al., 1986) and secrete unmutated Ig (Jacob et al., 1991b; McHeyzer-Williams et al., 1993). TD antigens also induce a germinal center (GC) pathway involving somatic hypermutation, affinity maturation, and production of memory B cells and long-lived PCs (Jacob et al., 1991b; MacLennan and Gray, 1986; McHeyzer-Williams et al., 1991). Post-GC PCs have extended half-lives (McHeyzer-Williams and Ahmed, 1999), produce high-affinity antibody, and reside preferentially in the bone marrow (Benner et al., 1981). Memory B cells rapidly expand and differentiate into PCs in response to antigen rechallenge (MacLennan and Gray, 1986; McHeyzer-Williams et al., 2000). Memory response PCs produce high-affinity antibodies but appear to have short life spans (Driver et al., 2001; McHeyzer-Williams et al., 2000).

How each of these PCs develops in vivo remains unclear. Antigen-driven cell expansion precedes terminal differentiation in each case and cell cycle arrest is necessary for terminal PC generation (Tourigny et al., 2002). However, as opposed to B cells that become short-lived PCs, B cells that have been through the GC reaction may differ substantially in their terminal differentiation path. Two subtypes of nonsecreting memory B cells that exit the GC (Driver et al., 2001) and respond to antigen rechallenge (McHeyzer-Williams et al., 2000) have been observed. Both populations express somatically mutated BCR with evidence of affinity-based selection (McHeyzer-Williams et al., 2000) but differ in cell surface phenotype, propensity to form PCs, and proliferative capacity upon adoptive transfer (McHeyzer-Williams et al., 2000). CD138$^-$B220$^-$ memory B cells produced 25-fold more PCs, but 15-fold fewer cells were recovered after transfer compared to CD138$^-$B220$^+$ memory cells. This suggests a linear progression from post-GC CD138$^-$B220$^+$ memory B cells to CD138$^-$B220$^-$ pre-plasma memory B cells to terminally differentiated CD138$^+$B220$^{+/-}$ PCs; however, there is no genetic evidence for a developmental link between these subsets.

B lymphocyte induced maturation protein-1 (Blimp-1) was initially described as a "master regulator" of plasma cell differentiation (Turner et al., 1994). Not only was Blimp-1 mRNA induced during differentiation of BCL1 cells to an IgM secreting state, but enforced expression of Blimp-1 was sufficient to drive differentiation (Turner et al., 1994). Subsequent studies confirmed that enforced expression of Blimp-1 is sufficient to drive plasmacytic differentiation of B cells at an appropriate developmental stage (Lin et al., 2000, 2002; Piskurich et al., 2000; Schliephake and Schimpl, 1996; Shaffer et al., 2002). Furthermore, Blimp-1 is expressed in all PCs and in a small subset of germinal center B cells that have some plasma cell characteristics (Angelin-Duclos et al., 2000; Falini et al., 2000). These data are all consistent with an important role for Blimp-1 in plasma cell differentiation (reviewed in Calame, 2001; Calame et al., 2003).

Blimp-1 is a 98 kDa protein containing five zinc finger motifs, which confer sequence-specific DNA binding (Keller and Maniatis, 1992; Tunyaplin et al., 2000). The protein is a transcriptional repressor that associates with hGroucho (Ren et al., 1999) and histone deacetylases (Yu et al., 2000). Functional Blimp-1 binding sites have been identified on several direct targets including *c-myc, CIITA* promoter III, *Pax5*, *SpiB*, and *Id3* (Lin et

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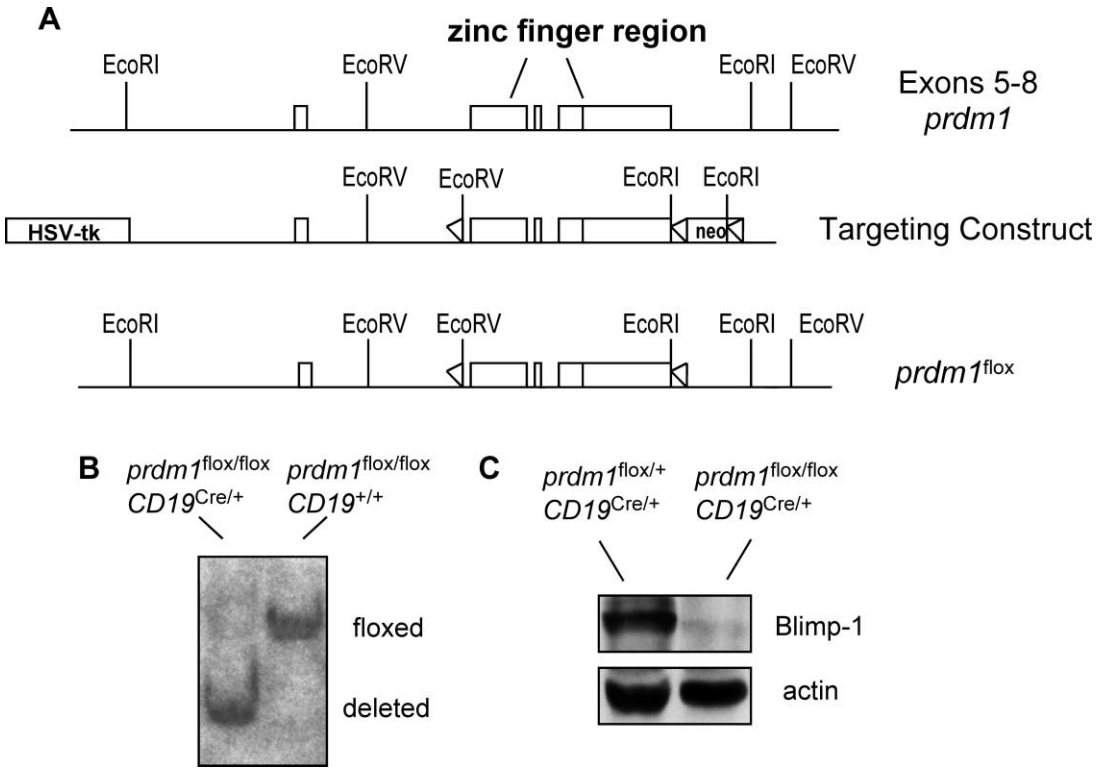


Figure 1. *Prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ Mice Have Efficient Deletion of *prdm1* in B Cells

(A) Strategy for generating an allele of *prdm1* with the zinc finger region flanked by LoxP sites (triangles). Part of the endogenous locus, targeting construct, and floxed allele after in vitro deletion of neo are shown.

(B) Southern blot analysis of B cell DNA to detect deleted and floxed *prdm1*.

(C) Immunoblots of cell lysates from splenocytes cultured in LPS for 3 days were blotted with mAb to Blimp-1.

al., 1997, 2002; Piskurich et al., 2000; Shaffer et al., 2002). In B cells, Blimp-1 regulates three large gene expression programs: cell cycle arrest, induction of Ig secretion, and inhibition of GC functions (Shaffer et al., 2002).

Although Blimp-1 is sufficient to drive plasmacytic differentiation, it is not known if Blimp-1 is required for plasmacytic differentiation. Gene targeting is the obvious way to approach this question. However, mice lacking *prdm1*, the gene encoding Blimp-1, die as early embryos (M. Davis, personal communication). This is probably because Blimp-1 is expressed in multiple embryonic cells, as early as day 7 (Chang and Calame, 2002). Therefore, we created mice in which the exons of *prdm1* that encode the zinc finger motifs of Blimp-1 are flanked by LoxP sites. Here we describe the phenotype of *prdm1*$^{flox/flox}$CD19$^{Cre/+}$ mice, which lack *prdm1* in their mature B cells. These mice have normal B cell development but cannot form PCs or secrete Ig normally. They are also defective in the formation of preplasma memory B cells.

Results

Conditional Deletion of *prdm1*

Exons that encode the zinc finger motifs of Blimp-1 were flanked with LoxP sites (Figure 1A). *Prdm1*$^{flox/flox}$ mice were crossed with mice expressing Cre recombinase under the control of the CD19 promoter (*CD19*$^{Cre/+}$) to achieve deletion of *prdm1* in B cells (Rickert et al., 1997). CD19 expression is B cell specific, beginning in early progenitors and continuing throughout development.

To determine the efficiency of *prdm1* deletion in B cells, B220^{+} splenocytes were purified from *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice and DNA was analyzed by Southern blotting. There is very efficient deletion of *prdm1* in B cells of the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice (Figure 1B). Similar results were seen using *prdm1*$^{flox/-}$*CD19*$^{Cre/+}$mice (not shown) so *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$and *prdm1*$^{flox/-}$*CD19*$^{Cre/+}$ mice were used interchangeably. To assess Blimp-1 protein, B220^{+} splenocytes were purified and stimulated ex vivo with LPS. Blimp-1 was almost undetectable in B cells from *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice although controls showed good expression (Figure 1C). Thus, we conservatively estimate at least 90% of the mature B cells from CD19$^{Cre/+}$*prdm1*$^{flox/flox}$ mice lack Blimp-1.

Severe Reduction in Ig Secretion and Plasma Cell Formation in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ Mice

The numbers and developmental subsets of B cells in the bone marrow and spleen of *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice appeared normal when analyzed by flow cytometry (not shown); however, serum Ig was significantly reduced in unimmunized mice (Figure 2A). To assess a TI response, *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ and littermate control mice were immunized with NP-Ficoll. Control mice showed normal increases in NP-specific serum IgM and

IgG3, but *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice had a greatly diminished serum Ig response (Figure 2B). NP-specific serum IgM, IgG1, and IgG2a were determined following immunization with NP-KLH precipitated in alum to study a TD response. The *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice showed a very large reduction in serum Ig of all 3 isotypes (Figure 2C). Failure to secrete Ig was also seen in a recall response elicited by a second immunization with NP-KLH in alum (Figure 2C).

ELISPOT assays were performed to determine if the decrease in serum Ig was due to a lack of Ig secreting cells in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice following immunization. In agreement with the decrease in serum IgM, there was a severe decrease in the number of NP-specific IgM secreting cells in the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice (Figure 2B, third panel, and 2C, second panel). We also determined the numbers of CD138$^+$ B220$^{+/-}$ PCs in the spleens of the immunized mice. After immunization with NP-KLH, control mice showed increased numbers of CD138$^+$B220$^{+/-}$ PCs in a pattern consistent with an early plasma cell response and a later post-GC response (Figure 2D). However, the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice had very few CD138$^+$B220$^{+/-}$ PCs at any time (Figure 2D).

Therefore, while Blimp-1 is not required for B cell maturation, it is required for formation of CD138$^+$B220$^{+/-}$ immunoglobulin-secreting PCs both early and late in a primary immune response. It is required for production of a response to both TI and TD antigens and for serum Ig in both a primary and recall response.

Pre-Plasma Memory B Cell Formation Is Defective in a Primary Response

A six-color flow cytometric strategy, including antigen binding and cell surface phenotype (Driver et al., 2001; McHeyzer-Williams et al., 2000), was used to quantify NP$^+$ subsets following IP immunization with NP-KLH in Ribi adjuvant. Baseline labeling in spleen and bone marrow before immunization using this strategy is negligible (Figures 3A, 3C, and 3D). As antibody can be cytophilic and passively adsorb to cells spuriously conferring antigen binding (Berken and Benacerraf, 1966; Boyden, 1960), we transferred NP-immune sera (2 doses of 200 μl) into naive mice with no change in NP binding (Figures 3A, 3C, and 3D). Thus, no passively adsorbed antibody in vivo interferes with the cellular quantification using the strategy presented here.

When immunized with adjuvant alone, negligible labeling was observed (Driver et al., 2001; McHeyzer-Williams et al., 2000). At day 7, the peak of early clonal expansion (Driver et al., 2001), total NP$^+$IgD$^-$ B cells (also PI$^-$CD4$^-$CD8$^-$) were equivalent in control and *prdm1*$^{flox/flox}$CD19$^{Cre/+}$ mice (Figures 3B and 3C, first panels). In contrast, there was a 95% reduction in total CD138$^+$B220$^{+/-}$ NP$^+$ PCs in the spleen at day 7 (Figures 3B and 3C, second panels; Figure 3C; $p = 0.004$). There was a substantial reduction in NP-specific PCs on day 14 in controls but PC numbers in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice were still significantly fewer (Figure 3C; 70% reduction, $p = 0.05$) as well as total CD138$^+$ cells in spleen sections (Figure 3E). Thus, antigen-specific clonal expansion was not dependent on Blimp-1; however, consistent with previous results (Figure 2), the development of both short-lived PCs (day 7) and post-GC long-lived PCs (day 14) was blocked in the absence of Blimp-1.

In contrast to the depletion of NP$^+$ PCs, there was an increase in the frequency (Figure 3B; 31 ± 0.7% and 62 ± 6.4% controls and *prdm1*$^{flox/flox}$CD19$^{Cre/+}$ respectively, $p = 0.004$) and total numbers (Figure 3C) of CD138$^-$B220$^+$ NP$^+$ B cells that expressed GL7 (a marker for GC B cells)(Han et al., 1997) at day 7 compared to preimmune animals (negligible numbers of total NP-specific cells see Figure 3C and NP-specific GL7$^+$ cells data not shown). This trend persisted to day 14 with respect to frequency among total NP$^+$ cells (32 ± 9% and 67 ± 10% control and *prdm1*$^{flox/flox}$CD19$^{Cre/+}$, respectively, $p = 0.03$) and total numbers (Figure 3C). A similar increase in total B220$^+$GL7$^+$ cells was observed in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice from day 3 to 20 following immunization (data not shown) with larger and more numerous GCs also seen with PNA staining of spleen sections (Figure 3E). Thus, B cells lacking Blimp-1 accumulate to higher numbers in the GC compartment without differentiating into long-lived PCs.

In control animals, a third subset of NP$^+$ B cells is evident as CD138$^-$B220$^-$ (Fig3B, second panel). This memory B cell subset expresses CD79b and not GL7 (data not shown), does not secrete antibody, and has undergone affinity maturation (Driver et al., 2001). Although not found in the GC, they probably originate there during the primary response (Driver et al., 2001). These cells will be referred to as pre-plasma memory B cells or B220$^-$CD79b$^+$ cells throughout this study. The majority of NP$^+$ cells that migrate and persist in the bone marrow after priming express the CD138$^-$B220$^-$CD79b$^+$ phenotype and are also considered pre-plasma memory B cells (Figure 3D, upper panels). Thus, bone marrow migration is a reliable indicator of pre-plasma memory B cell development. In the absence of Blimp-1, pre-plasma memory B cells fail to develop fully in the spleen. Although some NP$^+$ cells have lower B220 levels in the spleen (Figure 3B, lower middle panel, 3C total numbers), there is >90% reduction in the pre-plasma memory B cell compartment in the bone marrow on day 7 and >95% reduction on day 14 (Figure 3D; day 7 $p = 0.02$, day 14 $p = 0.04$). Thus, Blimp-1 is required for the complete development of pre-plasma memory B cells.

Memory B Cells Require Blimp-1 for Further Development and Plasma Cell Differentiation

The NP$^+$ memory B cell response 5 days after antigen rechallenge was also assessed (Figure 4). Total NP-specific memory B cells were very low on day 42 before rechallenge; however, a rapid and substantial expansion of NP$^+$ B cells was seen in both control and *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice (Figure 4A, first panels; Figure 4B, panel 1). In contrast, there was a profound alteration in memory B cell subsets that appeared in the absence of Blimp-1 (Figure 4A, second panels, and 4B, panels 2–4). Absence of CD138$^+$B220$^{+/-}$ NP$^+$ PCs was virtually complete with a 99% reduction in total cells (Figure 4A, second panels; Figure 4B, panel 2). Similar to the primary response, the CD138$^-$B220$^+$NP$^+$ compartment comprised the majority of the memory B cell response in the absence of Blimp-1. There were almost 10-fold more CD138$^-$B220$^+$NP$^+$ cells expressing the GC marker, GL7,

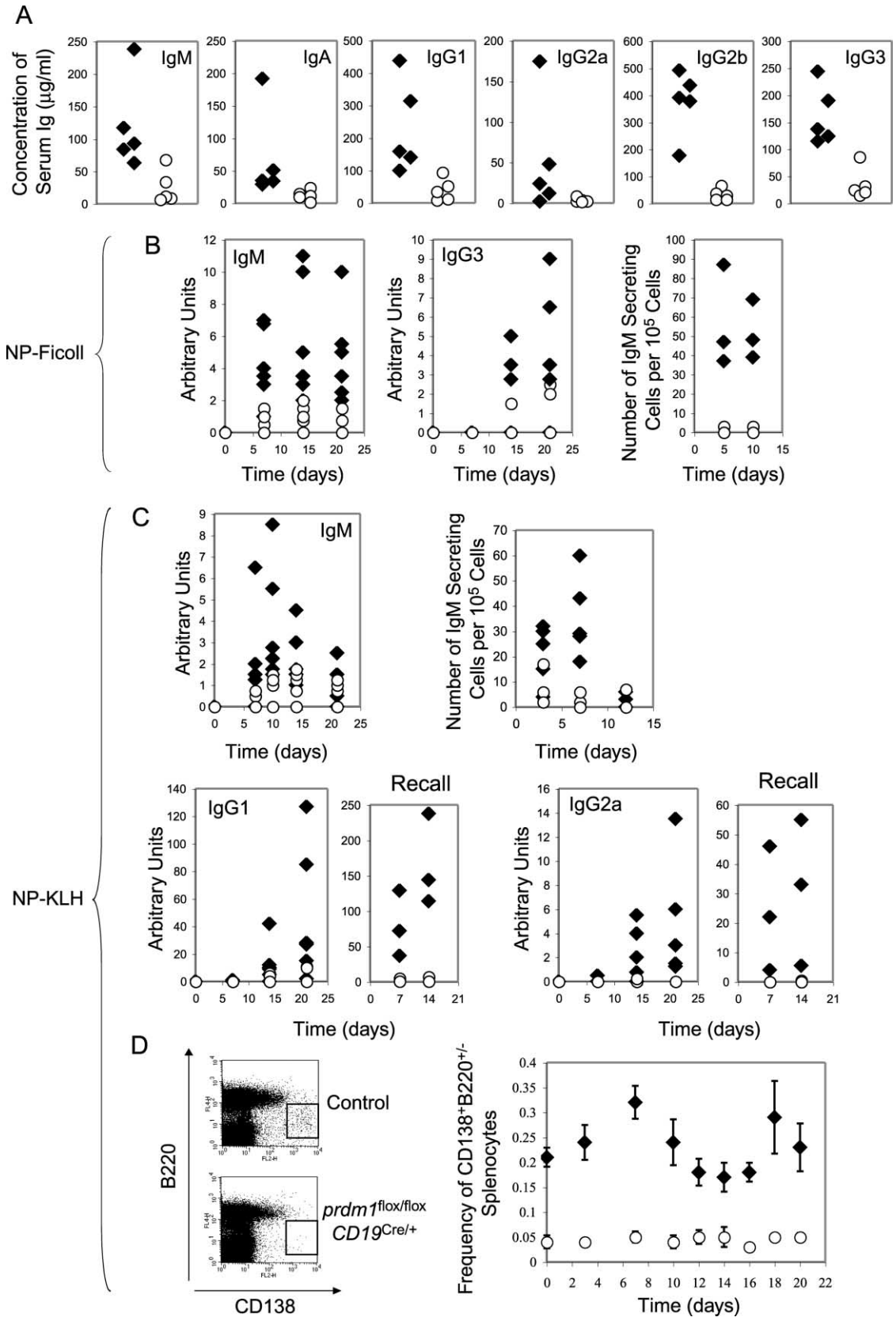


Figure 2. *Prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ Mice Do Not Develop PCs or Secrete Ig

(A) Serum from naive mice was analyzed for resting levels of IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 by ELISA. Mice were immunized with NP-Ficoll (B) or NP-KLH (C and D). Serum was analyzed by ELISA for NP-specific IgM and IgG3 (B) or NP-specific IgM, IgG1, and IgG2a (C).

in the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice compared to controls (Figure 4A, third panels, Figure 4B, panel 3). Pre-plasma memory B cells (CD138$^-$B220$^-$CD79b$^+$NP$^+$) were reduced 75% in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ spleens (Figure 4B, $p = 0.04$) and reduced 98% in the bone marrow ($p = 0.006$, data not shown).

Thus, the primary GC reaction resolved 42 days after initial challenge in the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice. However, similar to the primary response, the NP-specific memory response accumulated aberrantly in the GL7$^+$B220$^+$NP$^+$ compartment without producing pre-plasma memory B cells or PCs. Thus, Blimp-1 is clearly required for normal plasma cell development at all stages of the primary and memory response.

Prdm1$^{-/-}$ B Cells Cultured Ex Vivo with LPS Proliferate, but Fail to Induce μS mRNA or Secrete Immunoglobulin

When cultured ex vivo with LPS, murine splenic B cells proliferate and differentiate into Ig-secreting cells. To assess the proliferative capacity of stimulated B cells that lack Blimp-1, *prdm1*$^{-/-}$ and control B220$^+$ cells were cultured with LPS and viable cells were counted. At all time points after LPS treatment, there were more *prdm1*$^{-/-}$ than control cells (Figure 5A). When splenocytes were labeled with CFSE, after 2 days of LPS treatment, *prdm1*$^{-/-}$ cells had lower CFSE intensity than control cells (Figure 5B), indicating that the increased number of cells in the *prdm1*$^{-/-}$ cultures was due to increased proliferation. When propidium iodide staining was performed to quantitate apoptotic cells after stimulation with LPS, no significant difference was observed in the number of apoptotic cells in *prdm1*$^{-/-}$ and control cultures (not shown).

To analyze differentiation, secreted Ig and the number of Ig secreting plasma cells was determined in the LPS cultures. Control B cells secreted large quantities of IgM and small amounts of IgG3 but *prdm1*$^{-/-}$ B cells secreted almost no IgM or IgG3 (Figure 5C). Also, there were IgM secreting cells and CD138$^+$ cells in the control cultures, but almost none in the *prdm1*$^{-/-}$ cultures (Figures 5D and 5E). Therefore, even after activation with LPS, *prdm1*$^{-/-}$ B cells are unable to differentiate into CD138$^+$ cells or secrete Ig, recapitulating ex vivo the defects observed in vivo.

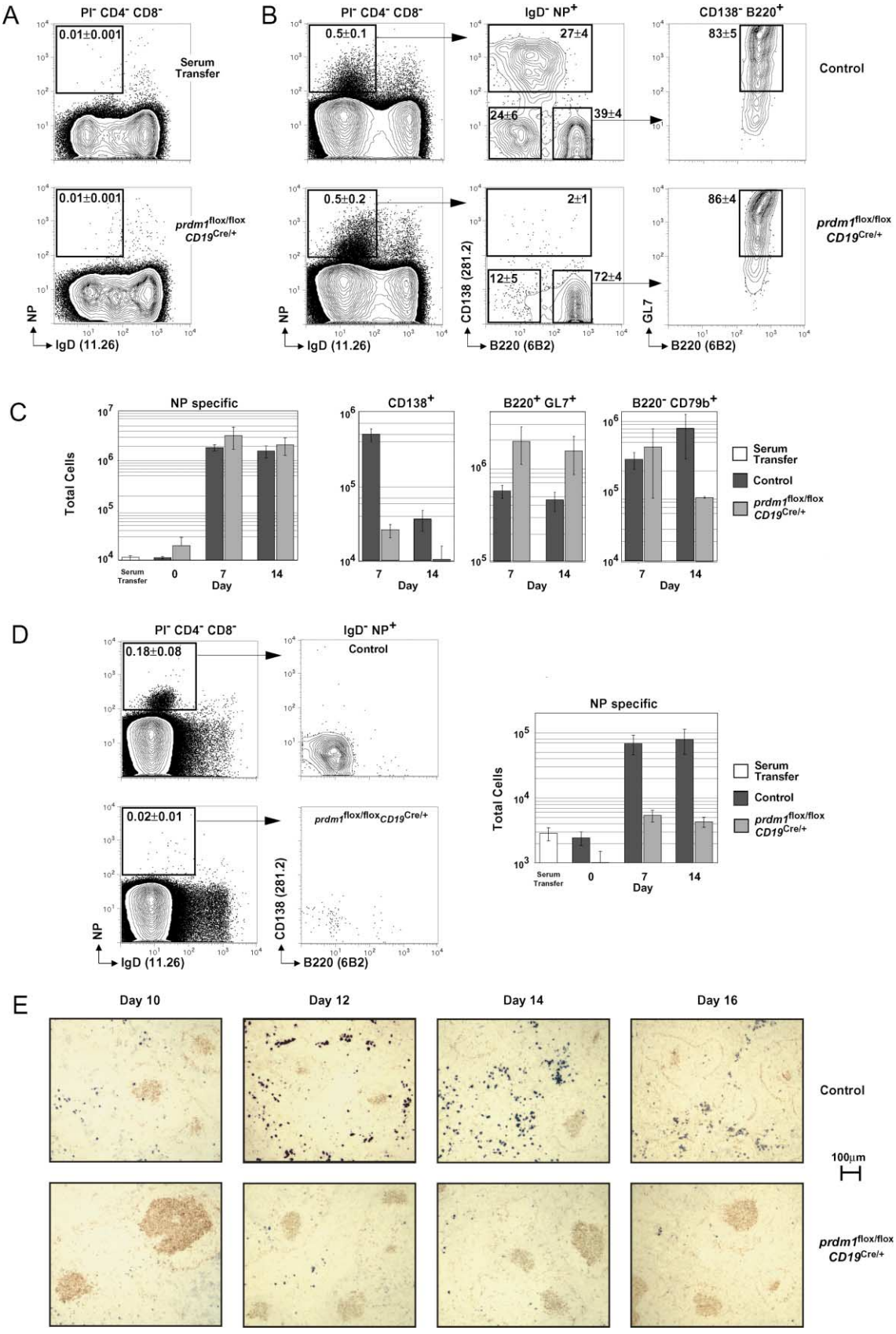
To investigate the mechanism responsible for the block in Ig secretion in *prdm1*$^{-/-}$ B cells, IgM protein in *prdm1*$^{-/-}$ and control cells was assayed by immunoblotting following culture with LPS. Cell lysates from *prdm1*$^{-/-}$ and control B cells contained similar amounts of the membrane form of μ heavy chain (μM), but control cells had more than 10-fold higher amounts of secreted μ (μS) than the *prdm1*$^{-/-}$ cells (Figure 5F). When μM and μS mRNA were analyzed by semiquantitative RT-PCR (Figure 5G), at least a 16× reduction in μS mRNA was observed in *prdm1*$^{-/-}$ B cells. We have also noted that *prdm1*$^{-/-}$ B cells are defective in induction of Ig light chain mRNA (A. Shaffer and M.S.-S., unpublished data). Thus, both the induction of Ig mRNA and the switch from μM to μS mRNA are defective in *prdm1*$^{-/-}$ B cells.

Blimp-1 and XBP-1 Have Separate Roles in Plasmacytic Differentiation

The activator XBP-1 is the only other transcription factor known to be required specifically for plasmacytic differentiation (Reimold et al., 2001). Splenic B cells lacking XBP-1 have normal levels of Blimp-1 following ex vivo stimulation, demonstrating that Blimp-1 cannot drive plasmacytic differentiation in the absence of XBP-1 and suggesting that Blimp-1 acts before XBP-1 in plasma cell development (Reimold et al., 2001). Consistent with this idea, Blimp-1 can induce XBP-1 mRNA indirectly by repressing *Pax5* (Lin et al., 2002; Shaffer et al., 2002). We analyzed XBP-1 mRNA and protein in B cells lacking Blimp-1. Semiquantitative RT-PCR showed that XBP-1 mRNA was strongly induced in LPS treated controls, but not in *prdm1*$^{-/-}$ cells (Figure 6A). Both unprocessed and processed forms of XBP-1 (Calfon et al., 2002; Iwakoshi et al., 2003; Lee et al., 2002; Yoshida et al., 2001) were induced in control cultures; however, minimal unprocessed and no processed XBP-1 was detected in *prdm1*$^{-/-}$ cultures (Figure 6B). Therefore, B cells without Blimp-1 fail to induce XBP-1 mRNA normally and are defective in their ability to make processed XBP-1 protein.

Since XBP-1 is required for plasma cell formation and because XBP-1 is not induced normally in the absence of Blimp-1, we wondered if the critical role for Blimp-1 in plasmacytic differentiation was *solely* induction of XBP-1. To investigate this possibility, LPS treated *prdm1*$^{-/-}$ splenic B cells were infected with bicistronic retroviruses expressing either Blimp-1 or XBP-1 (processed form) and YFP. YFP$^+$ cells were analyzed by flow cytometry for CD138 expression and by ELISPOT for IgM secretion. LPS treated *prdm1*$^{-/-}$ cells expressing exogenous Blimp-1 induced surface CD138 and secreted IgM, demonstrating that Blimp-1 expressed from a retrovirus can complement the *prdm1*$^{-/-}$ defect (Figures 6C and 6D). However, *prdm1*$^{-/-}$ cells infected with the XBP-1 virus were not able to induce CD138 or normally secrete IgM (Figures 6C and 6D). To confirm that functional XBP-1 was present in these cells, semiquantitative RT-PCR was performed on YFP$^+$ cells to amplify XBP-1 and DnaJ mRNA. *DnaJ* expression is activated by XBP-1 in B cells (Lee et al., 2003). In *prdm1*$^{-/-}$ cells infected with virus expressing XBP-1, and not in cells infected with control virus, XBP-1 mRNA was increased (Figure 6E). DnaJ mRNA was also induced in cells expressing XBP-1 (Figure 6E), providing evidence that the expressed XBP-1 was functional. These experiments demonstrate that Blimp-1 is fully able to rescue a defi-

Splenocytes were harvested at indicated days to quantitate NP-specific IgM secreting cells by ELISPOT (B and C). A secondary immunization with NP-KLH was given at least 6 weeks after priming and serum was analyzed by ELISA for NP-specific IgG1 and IgG2a (C). (D) On indicated days after NP-KLH, splenocytes were stained for B220 and CD138. Representative CD138 and B220 levels for day 7 post-NP-KLH are shown for *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ and control. The frequency of CD138$^+$B220$^{+/-}$ cells was determined. Each time point represents the average and SEM of at least three mice. For all panels, control mice are represented by filled diamonds and *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ by open circles.


A
PI- CD4- CD8-
0.01±0.001
Serum Transfer
0.01±0.001
prdm1flox/flox CD19Cre/+
NP
IgD (11.26)
B
PI- CD4- CD8-
0.5±0.1
IgD- NP+
27±4
24±6
39±4
CD138- B220+
83±5
Control
0.5±0.2
2±1
12±5
72±4
CD138 (281.2)
B220 (6B2)
86±4
GL7
prdm1flox/flox CD19Cre/+
C
NP specific
Total Cells
CD138+
B220+ GL7+
B220- CD79b+
Serum Transfer
Control
prdm1flox/flox CD19Cre/+
Day
D
PI- CD4- CD8-
0.18±0.08
IgD- NP+
Control
0.02±0.01
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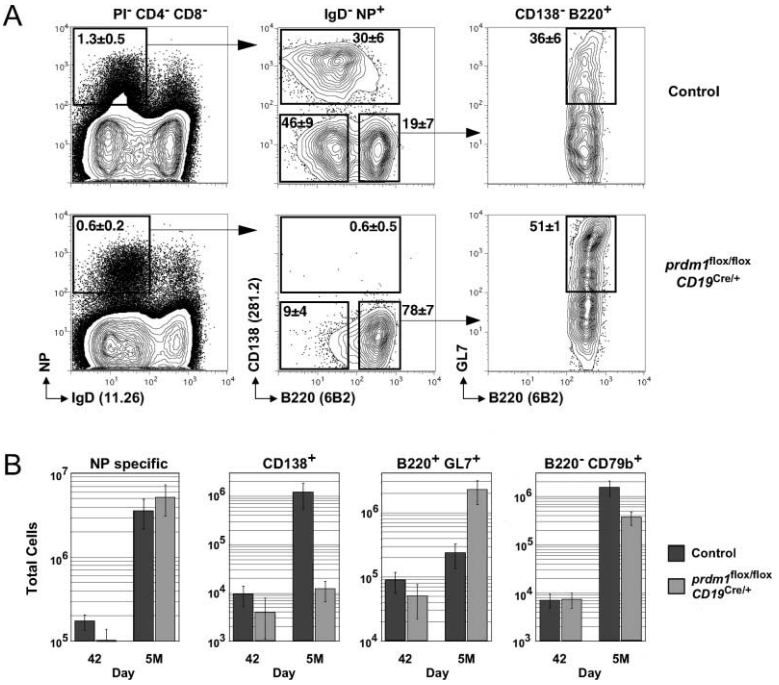

Figure 4. Memory B Cell Responses Require Blimp-1

Mice were immunized with NP-KLH in Ribi, rested for 6 weeks, and reimmunized with antigen in adjuvant. Splenocytes harvested before immunization at day 42 primary and 5 days after antigen recall, stained as described in Figure 3. (A) NP and IgD levels of $CD4^-CD8^-$ PI^- cells harvested from spleen on day 5 after recall (first two panels) with small box insert outlining NP^+IgD^- events. CD138 versus B220 levels on $PI^-CD4^-CD8^-$ NP^+IgD^- cells (second two panels). GL7 levels on $CD138^-B220^+$ NP-specific cells (third two panels), mean frequency ± SEM, n = 3. Controls displayed in upper panels and *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ lower panels. Mean frequency ± SEM, n = 3 across separate animals. (B) Cell numbers in spleen for each group at day 42 primary and day 5 memory: NP-specific cells ($PI^-CD4^-CD8^-NP^+IgD^-$), $CD138^+$ cells (NP-specific and $CD138^+B220^{+/-}$), $B220^+$ $GL7^+$(NP-specific and $CD138^-B220^+GL7^+$), $B220^-CD79b^+$ (NP-specific and $CD138^-B220^-CD79b^+$). Mean frequencies ± SEM, n = 3.

ciency in *prdm1*, but XBP-1 cannot compensate for a lack of Blimp-1. Thus, because XBP-1 is not sufficient to drive plasmacytic differentiation in the absence of Blimp-1, Blimp-1 must have other critical targets in plasmacytic differentiation besides XBP-1.

Discussion

Requirement for Blimp-1 in Plasma Cell Formation and Ig Secretion

Blimp-1 is known to be *sufficient* to drive terminal differentiation of B cells into Ig secreting PCs (Lin et al., 2000, 2002; Piskurich et al., 2000; Schliephake and Schimpl, 1996; Shaffer et al., 2002; Turner et al., 1994). The results presented here provide unequivocal evidence that Blimp-1 is also *required* for plasmacytic differentiation and Ig secretion. This is consistent with studies where expression of a blocking form of Blimp-1 inhibited Ig secretion by B cells cultured ex vivo (Shaffer et al., 2002). However, mice expressing a blocking form of Blimp-1 did not have defective plasma cell differentiation, presumably because endogenous Blimp-1 was not fully inhibited (Angelin-Duclos et al., 2002). Although previous studies using cultured B cells treated with antisense Blimp-1 oligonucleotides suggested that Blimp-1 was required for TI, but not TD responses (Soro et al., 1999), the phenotype of *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice establishes a role for Blimp-1 in response to both types of antigens. Mice lacking Blimp-1 in their B cells were unable to mount a normal humoral response to either a TI antigen or to the TD form (Figures 2–4). In the TD response, both early and post-germinal center responses were defective, as was a recall response to secondary challenge (Figures 2–4). However, some *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice did have a small number of PCs and/or serum Ig following immunization. At present we cannot distinguish between the possibility that a small fraction of B cells did not delete *prdm1* or the existence of a minor, Blimp-1-indepedent pathway for PC differentiation.

Finding that *prdm1*$^{-/-}$ B cells are hyperproliferative (Figure 5) confirms a role for Blimp-1 in cell cycle arrest and suggests that Blimp-1 is important for limiting the number of divisions plasmablasts undergo prior to terminal differentiation. This is consistent with microarray

Figure 3. *Prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ Mice Have Defective Development of Antigen-Specific PCs and Pre-Plasma Memory B Cells

(A) NP and IgD levels on $CD4^-CD8^-$ PI^- spleen cells before immunization. Upper panel presents a wt C57BL/6 that had received 2 × 200 μl NP-immunized serum IV before analysis and lower panel presents *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ animals. Inserts outline the region used for estimation of NP^+IgD^- cells with mean frequencies ± SEM across three separate animals depicted.

(B) NP and IgD levels on $CD4^-CD8^-$ PI^- cells day 7 after NP-KLH immunization (first two panels). Small box insert outlines NP^+IgD^-. CD138 versus B220 on $PI^-CD4^-CD8^-NP^+IgD^-$ cells (second two panels). GL7 levels on $CD138^-B220^+$ NP-specific cells (third two panels). Controls displayed in upper panels and *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ lower panels. Mean frequencies ± SEM, n = 3.

(C) Total NP-specific B cell numbers in spleen across day 0 (with serum transfer) and day 0, 7, and 14 after NP-KLH immunization (three mice/group) total NP-specific cells ($PI^-CD4^-CD8^-NP^+IgD^-$), $CD138^+$ cells (NP-specific and $CD138^+B220^{+/-}$), $B220^+$ $GL7^+$(NP-specific and $CD138^-B220^+GL7^+$), $B220^-CD79b^+$ (NP-specific and $CD138^-B220^-CD79b^+$).

(D) NP versus IgD levels on bone marrow harvested from day 7 immunized mice (first panels) mean frequencies ± SEM, n = 3 inserted. CD138 versus B220 on PI^-CD4^-NPTIgD$^-$ cells (second two panels). Total NP-specific cells in bone marrow (second panels) from two femurs mean ± SEM across three separate mice for day 0 (with serum transfer), day 0, 7, and 14 after NP-KLH immunization.

(E) Spleens from mice immunized with NP-KLH were stained for PNA (red) and CD138 (blue) 10, 12, 14, and 16 days after immunization.

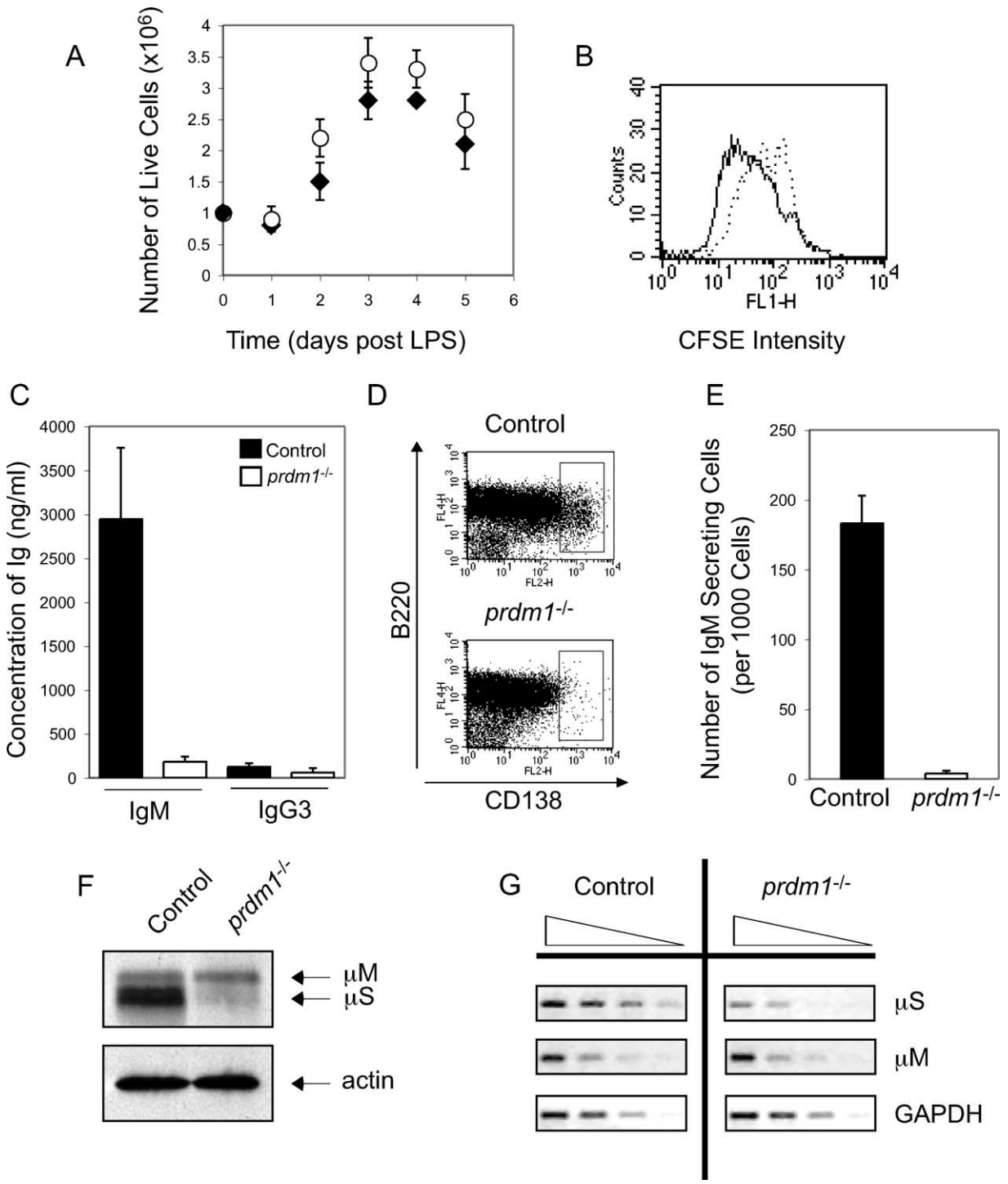

Figure 5. Upon Stimulation with LPS, *prdm1*$^{-/-}$ B Cells Proliferate but Do Not Become PCs, Secrete Ig, or Induce μS RNA or Protein

(A) Average number and SEM of viable cells during culture of B220^{+} cells with LPS (*prdm1*$^{-/-}$, open circles; control, filled diamonds). (B) Splenocytes incubated with CFSE, then cultured with LPS for 2 days, were stained with B220. The CFSE intensity of B220^{+} cells is shown (*prdm1*$^{-/-}$ B cells, solid line; control, dotted line; representative of four experiments). (C) Cell supernatants from LPS treated splenocytes were assayed for secreted IgM and IgG3 by ELISA. Splenocytes cultured in LPS for 4 days were stained for B220 and CD138 (D) or analyzed by ELISPOT to detect IgM secreting cells (E). (F) Cell lysates from splenocytes cultured for 4 days in LPS were analyzed by immunoblot to detect μM and μS protein. By day 2, cultures are at least 90% B220^{+} (data not shown). (G) Semiquantitative RT-PCR was used to detect μM and μS mRNA in LPS treated B220^{+} splenocytes.

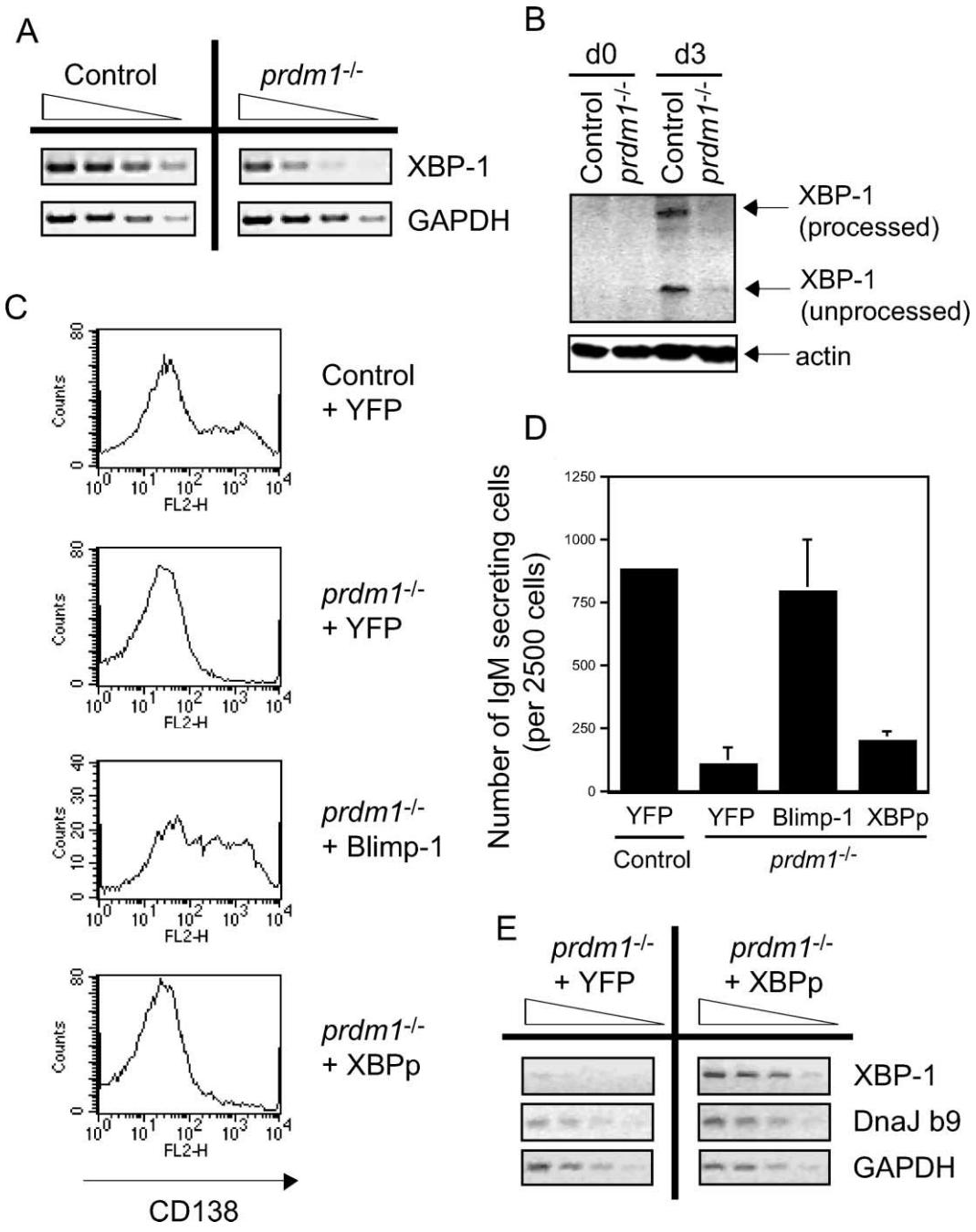

Figure 6. XBP-1 Is Not Induced or Processed in *prdm1*$^{-/-}$ B Cells but Exogenous XBP-1 Is Not Sufficient to Rescue Defective CD138 Expression and Ig Secretion in *prdm1*$^{-/-}$ B Cells

(A) RNA was harvested from B220^{+} splenocytes cultured in LPS for 4 days to be used for semiquantitative RT-PCR amplification of XBP-1 and GAPDH.

(B) Whole cell lysates from LPS treated splenocytes were immunoblotted to detect XBP-1 protein.

(C, D, and E) LPS treated splenocytes were infected with a retrovirus expressing YFP, YFP and Blimp-1, or YFP and XBP-1p. After 3 more days of culture, B220^{+}YFP^{+} cells were analyzed for CD138 expression (C) and sorted for ELISPOT analysis to detect the number of IgM secreting cells (D). RNA was also extracted from YFP^{+} cells for semiquantitative RT-PCR detection of XBP-1, DnaJ b9, and GAPDH (E).

studies that identified a large Blimp-1-dependent program that represses proliferation (Shaffer et al., 2002) and with the fact that terminally differentiated PCs do not divide. The Blimp-1-regulated proliferation program includes repression of c-*myc, E2F-1*, and other genes necessary for cell division as well as induction of cdk inhibitors p21 and p18 (Lin et al., 1997, 2000; Shaffer et al., 2002). Induction of p18 is particularly important, since mice lacking p18 have a severe reduction in antibody-containing plasmacytoid cells (Tourigny et al.,

2002). It is also consistent with hyperproliferation of B cells expressing a blocking form of Blimp-1 (Angelin-Duclos et al., 2002). However, proliferation of *prdm1*$^{-/-}$ B cells does stop after 3 or 4 days of ex vivo culture and we have not yet observed B cell lymphomas in the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice. Thus, there appear to be Blimp-1-independent, as well as Blimp-1-dependent mechanisms for regulating proliferation of maturing B cells.

Multiple mechanisms could be responsible for the defect in Ig secretion in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice. Previous studies (Lin et al., 2002) showed that Blimp-1 represses *Pax5,* relieving Pax5-dependent repression of J chain, Ig, and XBP-1 transcription (Shaffer et al., 1997; Singh and Birshtein, 1993; Wallin et al., 1999). The *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice confirm Blimp-1-dependent induction of Ig (Figure 5; data not shown) and XBP-1 mRNA (Figure 6). B cells lacking Blimp-1 also failed to generate μS mRNA and protein (Figure 5). This suggests a previously unknown requirement for Blimp-1 in the developmentally regulated switch from membrane (M) to secreted (S) μ. Whether this switch depends on differential polyA site usage, splicing, or mRNA stability remains unresolved (Berberich and Schimpl, 1990; Peterson and Perry, 1989; Phillips et al., 2001; Yan et al., 1995) and it will be interesting to determine if Blimp-1 affects the activity of Cstf64 polyadenylation complexes (Takagaki and Manley, 1998) or factors such as U1A (Phillips et al., 2001) that affect μ mRNA processing and stability. Alternatively, Blimp-1 could be required for an event developmentally upstream of the μM to μS switch. Future studies using inducible Cre recombinase may be useful in addressing this issue.

The phenotype of the *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice establishes that Blimp-1 is required to drive the major portion of plasmacytic differentiation and that timely arrest of cell cycle and induction of Ig secretion, two critical components of this process, depend on Blimp-1. It rules out the existence of any other transcriptional regulator with functions that can replace those of Blimp-1 in this context.

Blimp-1 and XBP-1

XBP-1 is the only transcription factor other than Blimp-1 known to be uniquely required for plasma cell formation and Ig secretion (Reimold et al., 2001). Since B cells lacking XBP-1 express Blimp-1 normally (Reimold et al., 2001), Blimp-1 is not sufficient for plasmacytic differentiation without XBP-1. The defect in XBP-1 mRNA induction observed in *prdm1*$^{-/-}$ B cells (Figure 6) is consistent with Blimp-1 acting before XBP-1 and confirms earlier data (Lin et al., 2002; Shaffer et al., 2002) showing Blimp-1 induces XBP-1 mRNA. XBP-1 is also subject to posttranscriptional regulation. The unfolded protein response (UPR) activates IRE1 endonuclease-dependent processing of XBP-1 mRNA to generate mRNA encoding a more active and stable form of XBP-1 (Calfon et al., 2002; Lee et al., 2002; Yoshida et al., 2001), which is required for PC differentiation (Iwakoshi et al., 2003). Furthermore, Ig synthesis is necessary for the B cell UPR that activates XBP-1 mRNA processing (Iwakoshi et al., 2003). Since Blimp-1 is required for induction of μS mRNA and protein (Figure 5), the induction of Ig synthesis and subsequent UPR-dependent processing of XBP-1 mRNA is a second, indirect, way that Blimp-1 regulates XBP-1.

Accordingly, we asked if failure to induce and process XBP-1 mRNA was the sole reason *prdm1*$^{-/-}$ B cells were blocked for PC differentiation. Forced expression of XBP-1 (processed form) was unable to restore differentiation and Ig secretion to *prdm1*$^{-/-}$ B cells following LPS treatment (Figure 6), demonstrating that Blimp-1 is required to regulate genes in addition to XBP-1. Thus, although Blimp-1 is important for regulation of XBP-1, Blimp-1 and XBP-1 each regulate unique targets. For example, Blimp-1 suppresses proliferation whereas proliferation is normal in XBP-1$^{-/-}$ B cells (Reimold et al., 2001). Furthermore, Blimp-1 represses genes involved in germinal center B cell functions (Shaffer et al., 2002) and *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice have abnormally large germinal centers (Figures 3 and 4). However, in XBP-1$^{-/-}$ chimeras, germinal centers are normal (Reimold et al., 2001). Finally, μS mRNA levels are normal in XBP-1$^{-/-}$ B cells (Reimold et al., 2001). Therefore these functions may be specifically dependent on Blimp-1. With respect to XBP-1, few targets have been identified, but *Grp78* (Yoshida et al., 2001) and *DnaJ* (Lee et al., 2003) may be unique direct targets of XBP-1.

A Role for Blimp-1 in Post-GC Memory Cells

Primary exposure to antigen drives the development of PCs and long-lived, antigen-experienced memory cells (MacLennan and Gray, 1986; McHeyzer-Williams, 2003; McHeyzer-Williams and Ahmed, 1999). Upon resolving the cellular basis of the plasma cell defect in *prdm1*$^{flox/flox}$ *CD19*$^{Cre/+}$ mice, we revealed an unexpected role for Blimp-1 in B cell memory (Figures 3 and 4).

The cellular organization of immune memory in vivo remains poorly understood. T_H memory cells exist in at least two forms, referred to as "central" and "effector," based on recirculation patterns, functional plasticity, and the rapidity of response to antigen recall (Lanzavecchia and Sallusto, 2002). B cell memory may also exist within these functional divisions (McHeyzer-Williams, 2003). CD138^{-}B220^{+} memory B cells recirculate through spleen and peripheral LNs (McHeyzer-Williams et al., 2000), have greater proliferative capacity on adoptive transfer, but produce fewer PCs as expected of a central memory compartment. In contrast, CD79b^{+}B220^{-} pre-plasma memory B cells appear mainly in the bone marrow (McHeyzer-Williams et al., 2000), differentiate rapidly into PCs on antigen recall (McHeyzer-Williams et al., 2000), and may be capable of homeostatic conversion to PCs in the absence of antigen (Bernasconi et al., 2002; O'Connor et al., 2002). However, they are not PCs, do not secrete antibody spontaneously (McHeyzer-Williams et al., 2000), and do exhibit attributes of an effector memory compartment. Our data show that Blimp-1 is required for the complete development of the pre-plasma memory B cell compartment (Figures 3 and 4). While other models are not ruled out, the simplest explanation of the data is a linear progression of post-germinal center development as outlined in Figure 7.

In this model, the GC reaction in a primary response produces B220^{+} central memory B cells. These cells appear rapidly in the marginal zones of the spleen (Liu

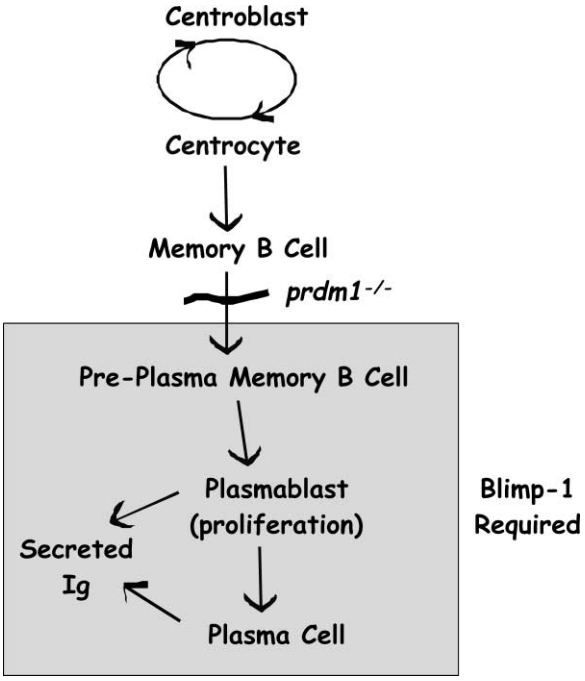

Figure 7. Model for Post GC B Cell Development
The gray box indicates stages that require Blimp-1.

et al., 1988), where they may encounter residual antigen and accessory signals (Balazs et al., 2002) to develop into pre-plasma memory B cells and/or PCs. These populations have a propensity to home to the bone marrow (Driver et al., 2001; McHeyzer-Williams et al., 2000) and persist in the absence of further antigen exposure (McHeyzer-Williams et al., 2000). Differentiation to PCs may also occur in the marrow (O'Connor et al., 2002). Localization and control of these changes remain speculative, but identifying a role for Blimp-1 provides new insight into their relationship and the basis for further experimentation.

It is not clear if $B220^+$ NP^+ B cells that persist in the absence of Blimp-1 are fully developed. They bind equivalent levels of NP-APC under saturating conditions (Figure 4; Total NP^+ B cells MFI 220 $\pm$ 13 and 310 $\pm$ 12; $B220^+$ NP^+ B cells MFI 280 $\pm$ 20 and 290 $\pm$ 16), suggesting expression of similar affinity BCR in control and *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice (McHeyzer-Williams et al., 2000). Without the capacity to produce antibody, it is difficult to test the function of Blimp-1-deficient B cells as bona fide memory cells on adoptive transfer (McHeyzer-Williams et al., 2000). It will also be important to evaluate the mutational status of the memory responders in *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice to assess more directly the activity of the primary GC reaction. Blimp-1-deficient B cells underwent rapid clonal expansion but then exhibited the same block in pre-plasma memory B cell and PC development as in the primary response (Figure 4). Interestingly, the selective pressures exerted by antigen rechallenge revealed even fewer residual PCs than in the primary response. The exaggerated levels of $GL7^+$ NP-specific cells at day 5 suggest extensive secondary GC formation that is an abnormal response to antigen recall (MacLennan and Gray, 1986). These aberrant memory responses may be manifestations of the same defect exhibited in the primary response, or they may indicate incomplete development of the $B220^+$ central memory compartment itself. In either case, an effective memory B cell response to antigen recall cannot be mounted in the absence of Blimp-1. Thus, in recall as well as primary responses, Blimp-1 is critical for memory and PC development.

Experimental Procedures

Generation of *prdm1*$^{Flox/Flox}$*CD19*$^{Cre/+}$ Mice

Exons 6-8 of *prdm1* (with 5′ LoxP site introduced by site-directed mutagenesis), 8 kb of 5′ sequence, 1 kb of 3′ sequence, a neomycin resistance gene (neo) flanked by LoxP sites, and an HSV-tk gene were inserted into pKS after replacing its multiple cloning site (Figure 1). R1 embryonic stem (ES) cells were electroporated and cultured using purified LIF (Hadjantonakis et al., 1999; Mereau et al., 1993; Nagy et al., 1993). Fialuridine and neomycin were used for negative and positive selection. Colonies were screened by PCR and Southern analysis for homologous recombination. Positive clones were injected into C57/BL6 blastocysts. Chimera progeny were crossed with mice expressing Cre ubiquitously to generate *prdm1*$^{+/-}$ mice (Lewandoski et al., 1997). Targeted ES clones were also transfected with a Cre vector and screened for deletion of neo. Appropriate clones were injected into C57/BL6 blastocysts. Chimera progeny were crossed to each other, to *prdm1*$^{+/-}$ mice, and to *CD19*$^{Cre/+}$ mice (Rickert et al., 1997) to generate *prdm1*$^{flox/-}$*CD19*$^{Cre/+}$ and *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ mice. *Prdm1*$^{flox/-}$*CD19*$^{+/+}$, *prdm1*$^{flox/flox}$*CD19*$^{+/+}$, *prdm1*$^{flox/+}$*CD19*$^{Cre/+}$, and *prdm1*$^{-/+}$*CD19*$^{Cre/+}$ were used as littermate controls.

Southern Blots

Genomic DNA was digested with EcoRI and hybridized with a 1 kb KpnI/EcoRV fragment of *prdm1* upstream of the deleted exons to detect endogenous, floxed, and deleted *prdm1* as 15, 13.5, and 10 kb bands respectively. The Cre recombinase gene was detected by BamHI digestion and probing with Cre sequence to reveal 6.5 and 2.5kb bands. To determine the deletion efficiency of *prdm1*, DNA from purified $B220^+$ splenocytes of *prdm1*$^{flox/-}$*CD19*$^{Cre/+}$, *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$, and control mice was analyzed.

Immunization

Prdm1$^{flox/flox}$*CD19*$^{Cre/+}$ and littermate control mice, 7- to 12-week-old, were immunized intraperitoneally (IP) with either 25 μg of (4-hydroxy-3-nitrophenyl)acetyl (NP)-Ficoll in 0.1 ml of PBS or 100 μg of NP-keyhole limpet hemocyanin (KLH) alum precipitated. For a recall response, the same dose of NP-KLH was given at least 6 weeks after the initial dose. For studies on NP-specific B cells, mice were immunized IP with 400 μg NP-KLH in Ribi adjuvant (Corixa). For the memory response, 6 weeks after primary immunization mice were reimmunized with 400 μg NP-KLH in Ribi adjuvant. Serum transfer experiments used pooled immune serum from NP-immune mice (3 $\times$ day 14, 3 $\times$ day 42, 5 $\times$ day 5 memory) with 200 μl injected into the tail vein of naive C57BL/6 mice, 3 days and 24 hr before analysis.

B Cell Culture

Splenocytes were suspended in NH_4Cl buffer (Sigma) to lyse red blood cells and plated (10^6 cells/ml) in RPMI with 10% FCS, β-mercaptoethanol, and gentamicin. Stimulation was with 10 μg/ml of lipopolysaccharide (LPS) (Sigma).

B Cell Purification

Freshly harvested splenocytes were incubated with APC conjugated α-mouse B220 (BD Pharmingen) in PBS with 1% BSA, 2 mM EDTA, and 2% FCS, washed, and incubated with goat α-rat IgG1 microbeads from Miltenyi. $B220^+$ cells were purified by positive selection using a MACS column (Miltenyi) according to manufacturer's instructions. Purity was assessed by flow cytometry.

Western Blots

Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting as described (Lin et al., 2000). Antibodies were monoclonal α-mouse Blimp-1 (Chang and Calame, 2002), monoclonal α-mouse β-actin (Sigma), goat α-mouse IgM (Roche), polyclonal rabbit α-mouse XBP-1 (Santa Cruz Biotechnology), goat α-mouse IgG1 conjugated to peroxidase (Roche), and α-goat IgG conjugated to peroxidase (Roche). For μS and μM blots, whole cell lysates were not centrifuged before SDS-PAGE. For XBP-1 blots, splenocytes were stimulated with 25 μg/ml LPS and, for 1 hr before lysis in 2×SDS loading buffer, treated with MG132 (10 μM, Sigma).

ELISA

To detect total IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in unimmunized mice, NP-specific IgM, IgG1, IgG2a, and IgG3 in immunized mice (6 control and 6 *prdm1*$^{flox/flox}$*CD19*$^{Cre/+}$ for primary response and 3 of each for recall) and secreted IgM and IgG3 in the cell supernatants of LPS stimulated splenocytes (3 days), enzyme-linked immunosorbent assays (ELISA) were performed as previously described (Angelin-Duclos et al., 2002). Additionally, for NP-specific Ig detection, plates were coated with NP(25)-BSA (Biosearch Technology) and for total IgA, with anti-IgA (BD Pharmingen).

ELISPOT

Enzyme-linked immunospot (ELISPOT) assays were performed (Angelin-Duclos et al., 2002) to detect NP-specific IgM secreting splenocytes from immunized mice and IgM secreting splenocytes after 4 days of LPS.

Flow Cytometry

For non-NP-specific experiments, splenocytes were prepared or cultured as above and stained in PBS with 1% BSA, 2 mM EDTA, 2% FCS, and 0.03% NaN_3 on ice for 45 min using APC α-mouse B220, FITC α-mouse B220, and PE α-mouse CD138 (all from BD Pharmingen). For analysis of NP$^+$ B cells, spleen and bone marrow were harvested as described (McHeyzer-Williams et al., 2000). Cells were stained at 2×10^6 cells/ml on ice for 45 min. The following antibodies were used for labeling cells and conjugated in the McHeyzer-Williams laboratory unless otherwise noted: Cy5PE-H129.19 (α-CD4; BD Pharmingen), Cy5PE-53-6.7 (α-CD8; BD Pharmingen), FITC-11.26 (α-IgD; a gift from F. Finkelman, Univ. Cincinnati Medical Center, Cincinnati, OH), biotin-11.26, allophycocyanin (APC)-conjugated NP (4-hydroxy-3-nitrophenyl), PE-281-2 (α-CD138; BD Pharmingen), Cy7PE-6B2 (α-B220, Caltag), FITC-GL7 (BD Pharmingen), biotin-HM79b (α-Igβ / CD79b), and streptavidin-Cy7APC (BD Pharmingen) as a second step revealing reagent. Cells were washed twice in PBS with 5% FCS and resuspended in 2 μg/ml propidium iodide (PI) with 5% FCS. Samples were analyzed using Cell Quest software on a FACS Vantage SE (BD Pharmingen). Standard analog compensation was used with Ominicomp correction between Cy5PE, Cy7PE, and APC, Cy7APC. Data were analyzed using FlowJo software (Tree Star). Profiles are presented as 5% probability contours with outliers.

CFSE Analysis

Splenic B cells prepared as above were incubated at 37°C for 15 min in 1 μM CFSE (Molecular Probes) in PBS. Cells were washed with media and plated. On multiple days after staining, cells were stained for B220 and analyzed by flow cytometry.

Semiquantitative RT-PCR

B220$^+$ cells cultured for 4 days with LPS or YFP$^+$ cells after retroviral infection (see below) were harvested and suspended in Trizol (Invitrogen Life Technologies). RNA was purified according to the manufacturer's instructions. cDNA was generated with AMV reverse-transcriptase (Invitrogen Life Technologies) according to the manufacturer instructions and PCR was performed on 4-fold dilutions of the cDNA using primers for XBP-1 and GAPDH (Shaffer et al., 2002), μS (5′-TCTGCCTTCACCACAGAAG-3′ and 5′-TAGCATG GTCAATAGCAGG-3′), μM (5′–GGCTTTGAGAACCTGTGGA-3′ and 5′-TTACAGCTCAGCTGTCTGT-3′), and DnaJ b9 (5′-AACACTCGGT CTAAGAAGC-3′ and 5′-ATCAGTGTATGTAGTAACC-3′).

Plasmids

Retroviral vectors pGC-YFP and pGC-Blimp-1-YFP have been described (Piskurich et al., 2000). pGC-XBPp-YFP was generated by the blunt ligation of XBP-1 cDNA from a plasmid that produces only processed XBP-1 protein, pFLAG.XBP1p.CMV2, into the pGC-YFP vector (Calfon et al., 2002).

Retroviral Transduction

The procedure for retrovirus vectors can be found at http://www.stanford.edu/group/nolan/index.html. For pseudotyped virus, 15 μg of retroviral vector, 15 μg of pSV-ψ$^-$-E-MLV, and 15 μg of VSV-G (pMD.G) was transfected into Phoenix cells. Viral supernatants were concentrated as described (Piskurich et al., 2000). Splenic cells treated with LPS (25 μg/ml) overnight were infected with concentrated virus stock (m.o.i. of 2–5). Three days after infection, cells were analyzed by FACS. YFP$^+$ cells were sorted by flow cytometry and used for ELISPOT analysis and semiquantitative RT-PCR.

Immunohistochemistry

Spleens were processed and stained as described (Angelin-Duclos et al., 2000) using the biotin-avidin-peroxidase system to detect PNA and the alkaline phosphatase system to detect CD138.

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