B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion

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B-1 cells produce circulating natural antibodies that provide "innate-like" protection against bacterial and viral pathogens. They also provide adaptive responses to blood and airborne pathogens. B lymphocyte–induced maturation protein 1 (Blimp-1) is a transcriptional repressor that is required for the formation of B-2–derived antibody-secreting plasma cells. In this study, we used mice lacking Blimp-1 in the B cell lineage to show that Blimp-1 is not necessary for the formation or self-renewal of B-1 cells but that Blimp-1 is required for normal immunoglobulin (Ig) secretion by B-1 cells. B-1 cells lacking Blimp-1 do not repress Pax5 mRNA and do not induce X-box binding protein 1, and μ secreted mRNA normally, showing that B-1 and B-2 cells both use a common pathway for Ig secretion. Blimp-1–deficient B-1 cells are also defective in providing early protection against influenza infection.

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their anatomical placement within the peritoneal cavity (PerC), spleen, and peripheral blood, and in their secretion of poly-specific, autoreactive antibodies (34, 35). In spite of such diverse and important roles for natural antibodies, the mechanisms that regulate antibody secretion by B-1 cells are poorly understood.

Our current molecular understanding of antibody secretion comes almost entirely from the investigation of B-2–derived plasma cells. Recent studies have revealed a network of transcription factors that regulate plasmacytic differentiation (36, 37). One principle player in this process is the transcriptional repressor, B lymphocyte–induced maturation protein 1 (Blimp-1; reference 38). Blimp-1 orchestrates a gene expression program that drives B cells to become plasma cells through the repression of genes involved in B cell proliferation, antigen presentation, germinal center reactions, BCR signaling, and B–T cell–cell interactions (39). Importantly, Blimp-1 also promotes the Ig secretion program (39–45). A crucial direct target of Blimp-1 for inducing the secretory program is Pax5, which encodes B cell lineage-specific activator protein and represses genes encoding Ig heavy chain, J chain protein, and X-box binding protein 1 (XBP-1; references 46–48). Blimp-1 relieves Pax5–dependent repression of XBP-1, which in turn functions as the proximal transcriptional activator for most of the genes necessary for the dramatic phenotypic changes in plasma cells associated with antibody secretion, including increases in cell and ER size, ribosomal and mitochondria number and function, and expression of numerous genes involved in the secretory pathway (49). Like Blimp-1, XBP-1 is required for plasma cell formation and antibody secretion (50). Blimp-1 is also required for processing of μ heavy chain transcripts to the secreted form (μS), although the molecular mechanism is not understood (42).

Recently, Tumang et al. (51) studied Bcl-6, Pax5, Blimp-1, and XBP-1 mRNA and protein levels in purified, Ig-secreting PerC B-1 cells and compared them to those of splenic B-2 cells activated with LPS to undergo plasmacytic differentiation.

Figure 1. Prdm1 gene deletion and phenotypic analysis of PerC and splenic B-1 cells. (A) Quantitative real-time PCR for prdm1 performed on genomic DNA from two purified CD19Cre/+prdm1Flox/Flox (open bars) and one littermate control (filled bar) PerC B-1 cell cultures. Primers were designed to amplify the floxed, but not the deleted, allele (see Materials and methods), and DNA loading was normalized to the peptidyl prolyl isomerase A gene. Percentages of prdm1Flox/Flox amplified DNA are shown. (B) Representative flow cytometry analysis from one purified CD19Cre/+ prdm1Flox/Flox B-1 cell culture used for deletion analysis in A, stained with antibodies against IgM and Mac-1. (C) Total PerC cells from CD19Cre/+ prdm1Flox/Flox mice (right) and littermate control (left) mice stained with antibodies against B220 and CD5. Upper gate is B-1a, and the lower gate is B-1b. Mean ± SEM, n = 7. (D) Bar graph shows averages and SEM of total PerC B-1 cells (IgM+Mac-1+), n = 7. (E) Total splenocytes stained with antibodies for CD43, B220, and CD5. CD43+ cells are shown. Mean ± SEM, n = 4.
to antibody-secreting cells. Similar to plasmacytic development of B-2 cells, these authors found that Bcl-6 and Pax5 mRNAs were decreased in B-1 cells whereas mRNAs encoding Blimp-1 and XBP-1 were not significantly elevated when compared with LPS-treated B-2 cells. Interestingly, XBP-1 protein levels were comparable to naive B-2 cells, and levels for Bcl-6, Blimp-1, and Pax5 were completely undetected in B-1 cells relative to naive and 2-d LPS-stimulated B-2 cells. These findings suggested that B-1 cells might use a different regulatory program for Ig secretion.

In a previous study, however, we demonstrated that naive mice lacking Blimp-1 in their B cells have dramatically reduced serum IgM, of which more than half comes from B-1 cells (7, 42). To follow up this observation, we sought to determine the nature of Blimp-1’s role in B-1 lymphocytes. Here we report that Blimp-1 is not required for B-1a or B-1b lymphocyte formation or for B-1 cell self-renewal. However, Blimp-1 is required for antibody secretion by B-1 cells, and in its absence, Pax5 mRNA is elevated while XBP-1 S mRNAs remain low compared with WT controls. Finally, we demonstrate that Blimp-1-deficient B-1 cells are also less effective at protecting reconstituted mice against influenza infection.

RESULTS

B-1 cell formation does not require Blimp-1

Blimp-1 is encoded by the prdm1 gene. CD19Cre/+ prdm1Flox/Flox mice and littermate controls were used to assess the role of Blimp-1 in B-1 cells. CD19Cre-dependent gene deletion is very efficient in splenic B cells (52), and deletion of the prdm1Flox/Flox allele in splenic B cells is nearly complete (42). Deletion of prdm1 in PerC B-1 cells was assessed by quantitative real-time PCR performed on genomic DNA prepared from PerC B-1 cells purified from CD19Cre/+ prdm1Flox/Flox and CD19Cre/+ prdm1Flox/Flox animals (Fig. 1 A). Primers were designed specifically to amplify floxed but not deleted prdm1 alleles, and samples were normalized using a control single copy gene. Less than 15% of the floxed allele was detected in CD19Cre/+ prdm1Flox/Flox B-1 cells. Because flow cytometry showed that the cells we analyzed were >70% Mac1+ IgM+ B-1 cells (Fig. 1 B), we conclude that prdm1 is efficiently deleted in B-1 cells of CD19Cre/+ prdm1Flox/Flox mice, hereafter referred to as conditional knockout (CKO) mice.

Flow cytometry was used to study B-1 cells in 6–10-wk-old CKO and control mice. No significant differences were observed in the frequency of PerC B-1a and B-1b subsets determined by staining for B220 and CD5 (Fig. 1 C). However, CKO mice had an increase in total cellularity in the PerC resulting in a 2.5-fold increase in total numbers of B-1 cells (Fig. 1 D). When total splenocytes were examined, no differences were seen in the frequency of CD5+ B220+ CD43+ splenic B-1a cells (Fig. 1 E) or in the overall cellularity of total splenocytes (not depicted). From these data, we conclude that Blimp-1 is not required for the formation or maintenance of B-1 cells in either the PerC or spleen. Moreover, we surmise that the absence of serum Ig observed in our initial study of naive CKO mice (42) was not due to the absence of B-1 cells.

Blimp-1-deficient B-1 lymphocytes are defective in antibody secretion

To determine directly the antibody-secreting ability of Blimp-1-deficient B-1 cells, ELISA assays were performed on supernatants from purified PerC B-1 cells cultured ex vivo. Although control B-1 cells secreted IgM in this setting, IgM secreted by B-1 cells derived from CKO mice was nearly undetectable (Fig. 2 A; WT, 756 ± 73 ng/ml; CKO, 53 ± 13 ng/ml).

Figure 2. Absence of Ig secretion in vitro and in vivo by CD19Cre+/prdm1Flox/Flox B-1 cells. (A) Anti-IgM ELISA performed on supernatants from B-1 cells after enrichment and in vitro culture. B-1 cells were plated at a density of 106 cells/ml for 4 d with no stimulation. Graph represents five control and eight CKO mice with SEM. (B) Summary of data from two immunohistochemical experiments for percentages of cytoplasmic IgM+ B-1 cells determined directly because the culture was assumed to be near 100% pure. (C) Bar graph showing data from one representative anti-IgM ELISA experiment for untreated (no bars) or LPS-treated (bars) samples. (D) Summary of data from two immunohistochemical experiments for percentages of cytoplasmic IgM+ B-1 (filled bars, 33.8 and 29.5%) and CD19Cre+/prdm1Flox/Flox B-1 (open bars, 9.2 and 10.2%), and 4-d LPS-treated splenic B-2 (gray bars, 39.1 and 37.9%) B cells. Percentages were determined by dividing the fraction of cytoplasmic IgM+ cells (WT, 68 IgM+/273 total and 114 IgM+/558 total; CKO, 23 IgM+/332 total and 32 IgM+/462 total) by the purity of the respective cultures (WT, 73.8 and 69.3% Mac1+ IgM+; CKO, 74.9 and 67.5% Mac1+ IgM+). The fraction of cytoplasmic IgM+ splenocytes (WT, 63 IgM+/161 total and 109 IgM+/297 total) was determined directly because the culture was assumed to be near 100% pure. (C) Bar graph showing data from one representative anti-IgM ELISA experiment for untreated (no bars) or LPS-treated (bars) control (filled bars) and CD19Cre+/prdm1Flox/Flox (open bars)-purified B-1 cell cultures. ELISA was performed as in A. (D) Anti-T15 ELISA against serum harvested from 6–10-wk-old CKO (filled squares, n = 8) and control (open squares, n = 6) mice. Filled bars represent the SEM. Units are OD405.
Similar results were obtained from sort-purified, cultured PerC B-1a and B-1b cells (not depicted).

To estimate the portion of cells secreting Ig in these B-1 cultures, we stained permeabilized cells for cytoplasmic Ig and calculated the fraction of cytoplasmic Ig⁺ B-1 cells. This analysis showed that in control B-1 cell cultures, 31.7% (average from two experiments) were secreting, as indicated by the presence of cytoplasmic Ig. In the KO cultures, this fraction was 3.2-fold lower or 9.7% (average from two experiments), consistent with the conclusion that Blimp-1 is required for Ig secretion by B-1 cells (Fig. 2 B and Fig. S1 A and B, which is available at http://www.jem.org/cgi/content/full/jem.20060411/DC1). For comparison, ~38.5% (average from two experiments) of LPS-activated B-2 splenocytes were found to be cytoplasmic Ig⁺ after 4 d in culture when similarly analyzed (Fig. 2 B and Fig. S1 C).

Treatment of PerC B-1 cells in vitro with LPS causes their proliferation (53), and in mice treated with LPS, PerC B-1 cells increase IgM secretion (28). When purified PerC B-1 cells were cultured for 3 d in LPS, the cells proliferated (on average, cell numbers doubled during the 3-d treatment) and greater than fivefold more IgM measured by ELISA was secreted into the cultures compared with untreated cultures. Similarly, when KO B-1 cells were treated with LPS they also doubled during the 3-d LPS treatment. However, the KO B-1 cells secreted 38-fold less IgM than LPS-treated control B-1 cells, although there was a small increase in IgM secretion comparing untreated and treated KO cultures (Fig. 2 C).

To study Ig secretion by B-1 cells in a more physiological context, we determined the relative serum levels of antibodies bearing the T15 idiotype in KO and WT mice. T15 idiotype antibodies recognize phosphorylcholine-containing self-antigens derived from oxidized lipids on apoptotic cells and atherosclerotic lesions (23, 54), provide protection against the pathogen Streptococcus pneumoniae (55), and are regarded as typical natural antibodies that are exclusively derived from B-1 cells (16). ELISA assays were performed for T15 antibodies in serum from WT and KO animals using a mixture of two rat anti-T15 antibodies, T139 and Tc54 (56). Relative T15 antibody levels in the sera of unimmunized KO mice were roughly equivalent to the level of detection of this assay in all but one of eight mice analyzed, whereas the T15 serum levels in all six unimmunized control animals were on average >2.8-fold above background (Fig. 2 D; WT, OD₄₀₅ = 0.404 ± 0.11; KO, OD₄₀₅ = 0.144 ± 0.07). Collectively, these data demonstrate that Blimp-1 is required for normal antibody secretion by B-1 cells both ex vivo and in vivo.

Ig secretion by B-1 cells depends on transcriptional regulators previously identified in B-2 cells

We next explored the molecular mechanisms underlying the requirement for Blimp-1 in Ig secretion by B-1 cells. In B-2 cells, direct repression of Pax5 leads to the derepression of the activator XBP-1, which then functions as the critical proximal regulator of a complex secretory program (42, 49). Furthermore, Blimp-1 is required for the processing of primary μS transcripts to the μS form of mRNA (42).

Quantitative RT–PCR was used to determine the steady-state levels of μS, Pax5, and XBP-1 mRNAs in KO and control B-1 cells. Purified PerC B-1 cells were analyzed with and without treatment with LPS for 3 d. Pax5 mRNA was higher in KO cells compared with WT cells, both without LPS and after LPS treatment. Furthermore, Pax5 mRNA decreased after LPS treatment in the WT but not in KO cells (Fig. 3 A; 2.9-fold difference between unstimulated KO/WT; 20.2-fold difference between LPS-stimulated KO/WT). These data provide evidence that Blimp-1 is required to repress Pax5 mRNA in B-1 cells. KO B-1 cells also had lower levels of XBP-1 mRNA without LPS treatment and failed to induce XBP-1 after LPS treatment compared with control B-1 cells (Fig. 3 B; 2.6-fold unstimulated WT/KO;
5.6-fold LPS-stimulated WT/CKO). Finally, μS was not expressed normally in untreated cells nor was it induced normally in LPS-treated CKO B-1 cell (Fig. 3 C; 3.0-fold unstimulated WT/CKO; 8.6-fold LPS-stimulated WT/CKO) transcripts. Thus, we conclude that Blimp-1 is required in B-1 cells for Pax5 repression and XBP-1 induction, as well as for formation of μS mRNA.

Blimp-1 is not required for self-renewal/homeostatic proliferation of B-1 cells

A unique feature of PerC B-1 cells, in contrast to B-2 cells, is their ability to regenerate the entire B-1 cell compartment. Adoptive transfer of peritoneal B-1 cells by i.p. injection into immunodeficient mice leads to the stable, long-term reconstitution of the PerC and IgA+ lamina propria B-1 cell pools, as well as restoration of natural IgM titers (7, 8). Peripheral B-2 cells, on the other hand, lack this ability and can only be generated from BM progenitors. To investigate a possible role for Blimp-1 in the self-renewal capacity of B-1 cells, total PerC cells from WT and CKO mice were harvested and adoptively transferred i.p. to 6–12-wk-old Rag1−/− mice. Recipient mice were killed 6–8 wk after transfer and the frequency of PerC B-1 cells was measured by flow cytometry. A small number of mice, receiving either WT or CKO B-1 cells, failed to reconstitute. Those in which reconstitution was <10% were excluded from the study. The half-life of B-1 cells has been reported to be between 38 and 56 d (57); therefore, recovery of >50% of donor B-1 cells after 6–8 wk indicates proliferation of the transferred B-1 cells. Representative flow cytometry analyses for IgM+Mac1+–stained PerC cells harvested from nonreconstituted, WT-reconstituted, and CKO-reconstituted Rag1−/− mice (Fig. 4 A) indicate that CKO cells can reconstitute Rag1−/− mice. CKO B-1 cells, as well as WT B-1 cells (Fig. 4 B; WT, 66.2 ± 17.1% recovered; CKO, 71.4 ± 13.9% recovered), proliferated and self-renewed in this experimental setting, and more total PerC cells were recovered from CKO-reconstituted Rag1−/− mice than WT-transferred Rag1−/− mice (Fig. 4 C; WT, 3.1 × 10^6 ± 0.55 total cells; CKO, 5.0 × 10^6 ± 0.81 total cells). In addition, T15 antibodies were detected in the sera of mice reconstituted with WT PerC cells at the time recipient mice were killed, demonstrating that transferred B-1 cells were functional (not depicted). Thus, we conclude that Blimp-1 is not required for the self-renewal/homeostatic proliferation of B-1 cells.

Blimp-1 is required for B-1 cells to protect against influenza virus infection in vivo

Baumgarth et al. (26) have elegantly demonstrated that both B-1 and B-2 cells are required for effective early immunity against influenza infection in mice. Specifically, B-1 cell–derived natural antibodies, present before infection, promote subsequent B-2 cell IgG2b responses and reduce mortality. This is probably because natural antibodies trap viruses and fix complement (58–60). Because Blimp-1 is required for natural antibody secretion by B-1 cells (Fig. 2), we hypothesized that B-1 cells lacking Blimp-1 would be defective in their ability to provide protection to influenza infection. To test this hypothesis, 4–6-wk-old irradiated muMT−/− mice were reconstituted i.v. with B-2 cells from BM from WT

![Figure 4. Normal self-renewal/homeostatic proliferation of Blimp-1−deficient B-1 cells.](image)

Equal numbers of total PerC cells from control and CD19^Cre+/prdm1Flox/Flox mice were transferred i.p. to Rag1-deficient hosts. 6–8 wk after transfer, PerC cells were harvested from recipient mice and the efficiency of B-1 cell reconstitution was determined by dividing the number of B-1 cells recovered by the number transferred. (A) Representative flow cytometry plots for anti-IgM– and Mac-1–stained PerC cells from Rag1−/− recipient mice either untreated (left) or receiving WT PerC cells (middle) or CD19^Cre+/prdm1Flox/Flox PerC cells (right) are shown. IgM+Mac1+ B-1 population is shown. (B) Bar graph showing the mean and SEM of the percentage of WT (filled bar) and CD19^Cre+/prdm1Flox/Flox (open bar) B-1 cells recovered for five WT and six CD19^Cre+/prdm1Flox/Flox samples. (C) Total PerC cells harvested from mice in B.
mice. Mice were also given PerC B-1 cells i.p. from either WT or CKO mice. B-2 cell reconstitution was confirmed by flow cytometric analysis performed for peripheral blood B220+ cells. 3 wk after reconstitution, mice were infected intranasally with an <LD50 dosage of A/WSN/33 influenza virus, and then monitored for 2 wk.

Weight loss was used as a criterion for susceptibility to influenza infection. In more than three independent experiments in which 27 mice were intranasally infected with influenza virus ranging from 4,500 to 7,000 PFU/g, we found only 1/13 WT-reconstituted mice (Fig. 5, top), but 9/14 CKO-reconstituted mice (Fig. 5, bottom) lost at least 30% of total body weight. Mice were killed when they lost 30% of their body weight to prevent excessive suffering, or on day 14 for analysis of the efficiency of B-1 cell reconstitution. Every mouse successfully reconstituted donor PerC B-1 cells as determined by flow cytometry analysis for surface IgM and Mac1 expression (not depicted). The increased susceptibility to influenza infection of mice receiving CKO B-1 cells demonstrates the physiological relevance of the requirement for Blimp-1 in antibody secretion by B-1 cells.

DISCUSSION

Blimp-1 is required for Ig secretion in B-1 cells

Our data reveal an essential role for Blimp-1 in antibody secretion by B-1 cells, both ex vivo and in vivo (Fig. 2). A requirement for Blimp-1 in antibody secretion by B-2 cells has been established previously (42). Earlier studies have shown that Blimp-1 is necessary for full induction of IgH, J chain, and XBP-1 mRNAs in B-2 cells, presumably due to derepression of these genes that are repressed by Pax5 (46–50), although recent papers disagree on whether or not Pax5 represses XBP-1 (61, 62). XBP-1 then functions as the proximal regulator of the Ig secretion program, inducing genes encoding proteins responsible for targeting proteins to the ER, cleavage of signal peptides, proper protein folding, degradation of misfolded proteins, and protein glycosylation, as well as proteins needed for ER and other organelle biogenesis and increased cell size (49, 63). In addition, Blimp-1 is required for the formation of μS mRNA, although the mechanistic basis for this requirement is not currently understood (42).

We therefore compared Pax5 mRNA repression, XBP-1 mRNA induction, and formation of μS mRNA in control versus CKO B-1 cells to determine if similar mechanisms were involved in Ig secretion by B-1 cells and B-2 plasma cells. We found that B-1 cells lacking Blimp-1 failed to repress Pax5 mRNA, failed to induce XBP-1 mRNA, and failed to form μS mRNA when compared with control B-1 cells (Fig. 3). These results provide strong evidence that the Blimp-1–dependent mechanisms we studied are important for Ig secretion in both B-1 and B-2 cells. This conclusion is also consistent with a previous study showing that mice lacking XBP-1 in their lymphocytes formed B-1 cells but failed to secrete IgM (50).

Why do spontaneously secreting B-1 cells have significantly lower levels of mRNA encoding Blimp-1 and XBP-1 compared with Ig-secreting B-2 cells, as reported by Tumang et al. (51)? The amount of IgM measured by ELISA in LPS-treated splenic B-2 cell supernatants is ~18-fold higher than that in cultures of purified B-1 cells (unpublished data).
Yet our data for purified B-1 cells in short-term culture (Fig. 2 B), and that of Tumang et al. for ex vivo–purified B-1 cells, show that a significant fraction (≈32 and ≈21%, respectively) of purified B-1 cells spontaneously secrete IgM. In addition, our data (Fig. 2 B and Fig. S1 C) indicate that a comparable fraction of LPS–stimulated B-2 cells are secreting, as measured by cytoplasmic Ig (≈39%). These data suggest that B-1 cells secrete less Ig per cell than B-2 cells. This conclusion is consistent with the ≈55% smaller spot sizes seen in B-1 cell ELISPOT assays, further demonstrating that B-1 cells secrete less IgM than do LPS–treated B splenocytes (51). Moreover, the morphology of B-1 cells is distinct from that of plasma cells. Although they have ample ER, B-1 cells lack the distinct arrays of rough ER characteristic of plasma cells (64). Thus, we suggest that although B-1 cells use the same regulatory mechanisms for Ig secretion, because they have less ER and secrete less Ig per cell, they may require lower amounts of Blimp-1 and XBP-1 mRNA and protein compared with B-2 plasma cells.

Our data clearly show that although Blimp-1 mRNA in B-1 cells is relatively low, it is nevertheless functionally important because it is required for normal Ig secretion. This conclusion is strengthened by the demonstration that Blimp-1–deficient B-1 cells do not secrete normal amounts of T15 natural antibodies (Fig. 2) and do not provide normal protection against influenza virus infection (Fig. 5).

Role of IgM in the formation of B-1 cells
Mice that cannot secrete IgM due to mutation in the µ-secreted exon and polyA sites have 1.5–2-fold increases in the frequency and total numbers of PerC B-1 cells (65, 66). The CD19C/C−/-prdm1Flox/Flox mice we studied have significantly reduced serum levels of all Ig isotypes including IgM (42). In spite of this, we did not observe an increase in the frequency of B-1a or B-1b cells in the PerC or in B220−CD5+CD43+B-1 cells in the spleen of these mice. There were, however, more total cells in the PerC of the CKO mice, resulting in a 2.5-fold increase in the total number of B-1 cells. Hence, our data support the idea that a lack of serum IgM feeds back to cause an increase in total B-1 cells in the PerC. The mechanism responsible for this effect remains obscure.

CKO B-1 cells, although deficient in Ig secretion, are normal in their ability to proliferate and self-renew, as demonstrated by their successful reconstitution of lymphopenic Rag1−/− and muMT− mice. We found no difference in the rate of recovery between CKO- and WT-transferred PerC B-1 cells after 6–8 wk after intraperitoneal transfer (Fig. 4), consistent with our previous observation that splenic B-2 cells from CKO mice proliferate well in response to LPS (42). Although we cannot formally rule out the possibility, we do not believe that CKO B-1 cell reconstitution was the result of preferential proliferation of CD19C/C−/-prdm1Flox/Flox B-1 cells that failed to delete prdm1. In addition to finding no differences in total cell numbers in our in vitro cultures, serum from WT–reconstituted Rag1−/− were found to have approximately ninefold greater T15 antibody levels than CKO–reconstituted mice (not depicted) and CKO–reconstituted muMT− mice were functionally inferior to WT–reconstituted mice upon challenge with influenza virus (Fig. 5).

The relationship between Ig secretion and cell division in B-1 cells
The B-1 cell compartment is heterogeneous and no single anatomical location or surface marker can define the entire population. B-1 cells are particularly uncharacterized in terms of two defining features: proliferation associated with self-renewal and Ig secretion. Further complexity is added by the fact that many B-1 cells are resting and perform neither function. Although in earlier studies Ig secretion was not detected in PerC B-1 cells (67, 68), Tumang et al. (51) showed by ELISPOT assay that ≈21% of naive, freshly sorted PerC CD5+B220+B-1 cells secreted IgM over 3 h. Our results on primary B-1 cells in short-term culture confirm this (Fig. 2, A and B, and Fig. S1 A). Many fewer PerC B-1 cells, however, are cycling than were found to be secreting: 2.5% of CD5+B-1 cell population. B-1 cells depleted of T cells and macrophages were found to be in cycle in vitro, and when the proliferative capacity of PerC CD5+B cells was determined in vivo, only 0.5–1.0% were in S phase (69, 70). Thus, it is not clear whether B-1 cells that secrete Ig have lost their proliferative capability, remain it, or, after a period of secretion, can revert to cells with proliferative potential.

In B-2 cells, plasmablasts are highly proliferative and also capable of secreting Ig. However, terminally differentiated plasma cells do not divide. Blimp-1 has been shown to repress multiple genes required for cell cycle entry, DNA replication, and cell division, and it is thought to be important for establishing/maintaining the postmitotic state of plasma cells (39, 43, 71–74). Nonetheless, dividing plasmablasts also express Blimp-1, demonstrating that Blimp-1 expression is not incompatible with cell division if the cells receive strong mitogenic signals (75, 76). Although additional studies will be necessary to learn if B-1 cells are fundamentally different from B-2 cells with respect to terminal differentiation to an Ig-secreting, nonproliferating state, we suspect that the low level of Blimp-1 mRNA in B-1 cells, compared with B-2 plasma cells, does not preclude Ig-secreting B-1 cells in the PerC from dividing when they receive appropriate signals. PerC B-1 cells may simultaneously retain both secretory and proliferative abilities, or they may alternate between secretory and proliferative states, but more data will be required to test this hypothesis. It will also be interesting to learn how overexpression of Blimp-1 might affect Ig secretion and proliferation of B-1 cells.

Overall, this study has shown that B-1 cells, like B-2 cells, require Blimp-1 and Blimp-1–dependent derepression of XBP-1 to secrete Ig. Antibodies derived from B-1 cells are important for immunity to mucosal and air-borne pathogens and are also implicated in autoimmune diseases, including systemic lupus erythematosus (77), Sjögren’s syndrome (78), and rheumatoid arthritis (79). Antibodies derived from B-2 cells are similarly critical for humoral immunity and involved
in autoimmunity. Understanding that both B-1 and B-2 cells use common mechanisms to secrete antibodies suggests that compounds designed to modulate the expression or activity of Blimp-1 or XBP-1 could affect both B-1 and B-2 cells and would be effective for either vaccine design or treatment of autoimmunity.

MATERIALS AND METHODS

Mice, cell transfers, and influenza infection. Prdm1<sup>Flox/Flox</sup> mice were crossed with CD19<sup>Cre/Cre</sup> mice to generate experimental (CD19<sup>Cre/+</sup> prdm1<sup>Flox/Flox</sup>) and control (CD19<sup>Cre/+</sup> prdm1<sup>Flox/Flox</sup>) mice. Rag<sup>1<sup>−/−</sup></sup> (B6.129S7-Rag<sup>1<sup>−/−</sup></sup>+/J) and muMT<sup>−</sup> (B6.129S2-Igh-6m1Cpr/J) were from The Jackson Laboratory. All mouse procedures were approved by Columbia University’s Institutional Animal Care and Use Committee. PerC cells were harvested in 4% FBS, 1% BSA in PBS, 3–5 × 10<sup>6</sup> PerC cells were resuspended in 1 ml PBS and transferred i.p. to Rag<sup>1<sup>−/−</sup></sup> or muMT<sup>−</sup> mice. The remaining PerC cells were stained with IgM and Mac-1 antibodies and analyzed by flow cytometry. For BM reconstitution, muMT<sup>−</sup> mice were lethally irradiated with 2× 700 rad separated by 4 h. Mice were rested overnight and then reconstituted via tail vein injection with 10<sup>7</sup> total BM cells in 200 ul PBS harvested from CD19<sup>Cre/+</sup> prdm1<sup>Flox/Flox</sup> control mice. Mice were fed water containing Baytayl (enrofloxacin) for the remainder of the experiment.

Influenza virus A/WSN/33 was provided by P. Palese (Mount Sinai School of Medicine, New York, NY). Influenza virus was cultured on Mardin-Darby Bovine Kidney cells in modified Eagle’s medium supplemented with 25 μg each of T139.2 and TC54.8 antibodies in PBS for 60 min, then blocked with a solution of 2% BSA in PBS overnight at 4°C. Wells were washed once with 0.05% Tween-20 in PBS and exposed to mouse serum (1:2 dilutions) for 60 min at 37°C. Wells were washed four times and incubated with a 1:500 dilution of goat anti-mouse IgG(H+L)-AP secondary antibody (SouthernBioTech) in 1% BSA in PBS for 60 min at 37°C. Four additional washes followed by development with 0.8 mg/ml of Sigma 104 phosphatase substrate (Sigma-Aldrich) in p-nitrophenyl phosphate buffer, and spectrophotometric measurements at OD<sub>405</sub> were performed.

Immunohistochemistry. Purified B-1 cells or splenocytes were seeded on slides by cytospin at 800 rpm for 5 min, air dried, fixed, and permeabilized with 1% paraformaldehyde plus 0.2% Tween-20 in PBS for 20 min. Egg white in PBS was incubated for 1 h to block, followed by incubation with 3% human serum (Sigma-Aldrich) plus 3% FBS plus 1% BSA in PBS for 20 min. Primary goat anti-mouse Ig(H+L) antibody (SouthernBiotech) was diluted at 1:5,000 in serum block and applied to slides overnight. The slides were washed for 45 min with TBST (50 mM Tris, pH 7.5, plus 0.2% Tween-20) and incubated with 1:100 diluted rabbit anti–goat IgG(H+L) alkaline phosphatase–conjugated secondary antibody (SouthernBiotech) for 1 h in serum block. Slides were washed as described above and developed with fast blue (Sigma-Aldrich) and naphthol ASBiphosphate (Sigma-Aldrich) in 100 mM Tris–HCl, pH 9.2. A Nikon Eclipse TE300 microscope and Openlab (Improvement) software were used for photographic analysis. Cells with darkly staining rings of cytoplasm or with darkly staining cytoplasmic caps were scored positive.

Quantitative real-time PCR. Quantitative real-time PCR was performed with a cycle of 50°C, 2 min; 95°C, 10 min; 95°C, 15 s; 60°C, 1 min; and 81°C, 20 s for 40 cycles, recording data at 81°C and using primers for the unprocessed form of XBP-1 (5′-AGCAGCTGAAGTGAGCCCT-3′) and primer for mouse β2 microglobulin (5′-AGCTGAGCATACGCGGC-3′). Purified B-1 cells or splenocytes were seeded on slides by cytospin as described above. Total RNA and cDNA were prepared from at least 0.15 × 10<sup>6</sup> purified B-1 cells by TrIZol and Superscript III reverse transcriptase according to the manufacturer’s instructions (Invitrogen). Genomic DNA was made by lysis of purified B-1 cells in 50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, and 12.5% SDS, followed by phenol/chloroform extraction and ethanol precipitation.

Statistics. Data were expressed as the mean ± SEM. Statistical significance was determined by a two-tail, unpaired Student’s t test.

Online supplemental material. Fig. S1 shows representative photographs and western blot results for purplicin expression of the anti–IgM ELISA assay and ELISA for IgM antibodies in the B10.BR B-1 culture. OpenLab (Improvement) software were used for photographic analysis. Cells with darkly staining rings of cytoplasm or with darkly staining cytoplasmic caps were scored positive.

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