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Different Kinetics of Blimp-1 Induction in B Cell Subsets Revealed by Reporter Gene

Kirsten A. Fairfax,* Lynn M. Corcoran,* Clare Pridans,† Nicholas D. Huntington,* Axel Kallies,* Stephen L. Nutt,* and David M. Tarlinton‡*

The transcriptional repressor Blimp-1 (B lymphocyte-induced maturation protein 1) has been described as a "master regulator" of B cell differentiation into Ab-secreting cells (ASCs). Although there is mounting evidence for the importance and necessity of Blimp-1 in plasma cell development, there is uncertainty as to the role it plays in B cell differentiation of B cell subsets and the way in which it may interact with other transcription factors such as Pax5 and Bcl6 during ASC differentiation. Using a mouse expressing GFP under the control of the Blimp-1 regulatory elements (Blimp-1GFP/+), we examined the kinetics of Blimp-1 up-regulation in purified B cell subsets following activation. B1 cells showed the most rapid and pronounced up-regulation of Blimp-1 in response to the mitogens tested, followed by marginal zone B cells and then conventional B2 cells. Interestingly, only B1 cells substantially up-regulated Blimp-1 expression in response to CpG. B1 cells secreted negligible Ig upon isolation but were able to up-regulate Blimp-1 and initiate Ig secretion within 28 h of stimulation. Also of interest, B1 cells have a transcriptional profile that is intermediate between a naïve B cell and an ASC, indicative of the semiactivated state of B1 cells. Transferred naïve Blimp-1GFP/+ B1 and B2 cells both gave rise to ASCs in the bone marrow, suggesting no intrinsic barriers to B1 cell entry into the long-lived ASC compartment.

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Abstract

B cells play a critical role within the immune system, providing protective humoral responses that contribute to the containment and elimination of pathogens. A number of mature B cell subsets have been identified based on their cell surface phenotype, anatomical localization, and functional properties (1, 2). The predominant B cell population found in the periphery is the B2 or follicular B cell. In addition to B2 cells, the spleen also contains marginal zone (MZ) B cells. These slightly larger B cells are found outside the marginal sinus marking the boundary between the white and red pulp of the spleen (3). MZ B cells are functionally distinct from B2 cells in that they proliferate and differentiate more rapidly than B2 cells in response to a variety of stimuli, both in vitro and in vivo (4–6). The final subset of mature B cells, B1 cells, reside predominantly in the peritoneal and pleural cavities, although small numbers are found in spleen and lymph nodes (7). B1 cell development requires a relatively narrow range of stimulation through the BCR, thought to be predominantly provided by self-Ags, and the population has the unique ability to self-renew as a mature cell (8) (reviewed in Ref. 2). Both MZ and B1 B cells have a skewed reactivity toward self and bacterial Ags and both have been shown in vivo to proliferate rapidly in response to bacterial Ags (5).

Ab-secreting cell (ASC) formation is correlated with the expression of a number of transcription factors, some of which have been shown recently to be critical for the production of Abs. The transcriptional repressor B lymphocyte-induced maturation protein 1 (Blimp-1) has been proposed to be the master regulator of ASC differentiation, because mice with Blimp-1-deficient B cells show vastly decreased Ab titers (9). This phenotypic trait, however, is not unique to Blimp-1-deficient animals; both X box-binding protein 1 (XBP-1)-deficient and IFN-regulatory factor 4 (IRF-4)-deficient mice also have severe defects in Ab production (10, 11). Conversely, molecules such as Pax5, Bcl6, and MTA3 are associated with the mature B cell phenotype and extinguishment of their expression is correlated with ASC formation (12, 13). Overexpression of Pax5 or Bcl6 with or without MTA3 in plasma cell lines resulted in a partial reversion toward a mature B cell phenotype with decreased Ig secretion, decreased expression of the plasma cell marker syndecan-1 (CD138), and re-expression of CD19 and HLA-DR (13, 14). The roles of the various transcriptional regulators of ASC differentiation have been elucidated in follicular B cells but little is known about their regulation in MZ and B1 B cells or the paths of activation that allow for the rapid production of Abs from these distinct B cell types.

It has been proposed that the B1 cell subset has the potential to spontaneously secrete Ab and to do so without apparent up-regulation of Blimp-1 (15). Surprisingly, Tumang et al. (15) also noted an absence of Pax-5 in peritoneal B1 cells. These results were unexpected and contrary to the predicted behavior of B cells, because Pax-5 is key in maintaining B cell identity (16) while Blimp-1 is required for ASC differentiation (9). This departure from the commonly accepted paradigm of the transcriptional regulation of B cell differentiation was intriguing and, as such, we
thought it important to explore the implications of these results. We did this using Blimp-1GFP+/ reporter mice that allowed B cell differentiation to be measured with precision on a single-cell basis. Our results show that upon isolation both peritoneal B1 and splenic B2 cells do not secrete Ig within the limit of resolution of our assays and that peritoneal B1 cells secrete Ab in a B1-1-dependent fashion. We found also that when taken directly ex vivo peritoneal B1 cells express Pax5 at a similar level as do splenic B2 cells and that Pax5 is down-regulated upon stimulation, which is in agreement with other recent data (17). We have also delineated the kinetics with which the different mature B cell subsets up-regulate the expression of Blimp-1 in response to in vitro conditions that mimic both T cell-dependent and T cell-independent stimulation and find distinct differences between the mature B cell subsets.

**Materials and Methods**

*Mice*

C57BL/6, BALB/c, CD19-/-, Rag1-/-, Pax5-/-, Blimp-1GFP+/ reporter, and Blimp-1GFP+/GFP mice were bred and maintained at the Walter and Eliza Hall Institute of Medical Research (Parkville, Australia). Construction of the Blimp-1GFP+/ reporter strain has been described as has the generation of Blimp-1GFP+/GFP reconstituted mice (18). All mice were used between 6 and 20 wk of age. All animal experimentation was in accordance with protocols approved by the Melbourne Health Animal Ethics Committee (Melbourne, Australia).

**ELISA and ELISPOT assay**

Ig levels were measured using ELISA as described (19) and ELISPOT as described (18). Plates were developed using 3-amino-9-ethylcarbazole for ELISPOT and ABTS for ELISA. ELISPOT and ELISA experiments were performed in duplicate and three times, respectively, using both C57BL/6 and BALB/C mice.

**Abs, flow cytometry, and cell sorting**

Single-cell suspensions were obtained by splenic disruption or peritoneal lavage and were handled at 4°C to minimize cell activation during handling. Cells were stained with a mixture of anti-CD19 (clone I3D) and either anti-CD21 (clone 7G6) and anti-CD23 (clone B3B4) (spleen) or anti-B220 (RA3-6B2) and anti-CD5 (53-7.3). All Abs were purified and conjugated in our own laboratory. Propidium iodide (1 µg/ml) was added to exclude dead cells. The legend to Fig. 1A describes the sorting procedure, although in some experiments CD19 was used in place of B220 for stimulation and find distinct differences between the mature B cell subsets.

**Intravenous injections**

Peritoneal B1 and splenic B2 cells were purified by sort ex vivo. Five hundred thousand B1 or B2 cells were injected i.v. per CD19-/- recipient. Six days later spleens and bone marrow were collected, single cell suspensions were made, erythrocytes were removed, and the samples were analyzed for the percentage and number of ASCs (GFP+/CD138+) in each organ.

**Results**

**Measuring Blimp-1 expression**

The importance of Blimp-1 in ASC formation has been demonstrated in several circumstances (9, 18). We have also identified and sorted from Blimp-1GFP/+ reporter mice that allowed B cell differentiation to be measured with precision on a single-cell basis. Our results show that upon isolation both peritoneal B1 and splenic B2 cells do not secrete Ig within the limit of resolution of our assays and that peritoneal B1 cells secrete Ab in a B1-1-dependent fashion. We found also that when taken directly ex vivo peritoneal B1 cells express Pax5 at a similar level as do splenic B2 cells and that Pax5 is down-regulated upon stimulation, which is in agreement with other recent data (17). We have also delineated the kinetics with which the different mature B cell subsets up-regulate the expression of Blimp-1 in response to in vitro conditions that mimic both T cell-dependent and T cell-independent stimulation and find distinct differences between the mature B cell subsets.

**Whole cell lysates from 1.5 x 10⁶ B cells were prepared, separated by electrophoresis, and transferred to membranes as described (20). To verify equivalent protein loading, blots were stripped and reprobed with anti-actin (Santa Cruz Biotechnology). The anti-Blimp-1 mAb was used (18). Primary Abs were revealed using anti-rat-HRP (BD Pharmingen) and anti-goat-HRP (Santa Cruz Biotechnology). For Western blot analyses, B cells were stimulated with 20 µg/ml LPS.

**Quantitative real-time RT-PCR**

Total RNA was isolated from cells using an RNaseasy mini kit (Qiagen) and cDNA was synthesized using the SuperScript III first strand synthesis system (Invitrogen Life Technologies). Quantitative real-time RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) on an ABI PRISM 7900HT cycler (Applied Biosystems). Expression of individual genes was normalized using Hprt, Blimp-1 primers were obtained from Qiagen (Quintet Primer Assay). Other primer sequences used were for the following: Mad1 (5'-GAAAGCTGTTCTTCAAGAACCTGTTGATGATC) and 5'-GCCGCTCAATATCTGACGACCTGTTGATGATC; Bcl-6 (5'-GCCGGCTCAATATCTGACGACCTGTTGATGATC and 5'-CCAGCACTCCTCTTGATGG); Hprt1 (5'-GTTGGAAGATGATC and 5'-GTTGGAAGATGATC); Irf4 (5'-GAAAGCCCAAGGCTGCTGTTGATGATC and 5'-GAGGCCATGTTAATCAACCTGTTGATGATC); Irf5 (5'-GAAAGCTGCTGAAGCTGCTGTTGATGATC and 5'-GAGGCCATGTTAATCAACCTGTTGATGATC).

**Cell culture**

B cells were cultured in RPMI 1640 supplemented with FCS (10%) and 2-ME (50 µM). The mitogens used for the in vitro stimulations for each of the cell subsets were LPS (2 or 20 µg/ml as indicated; Difco), CpG-1668P (phosphorothioate backbone) (200 nm; GeneWorks) and baculovirus-derived mouse CD40L (optimal concentration determined by B cell cultures), IL-4 (100 U; PeproTech), and IL-5 (5 ng/ml; a gift from Prof. N. Nicola, Walter and Eliza Hall Institute, Parkville, Australia). FITC-labeled beads were added at harvest time and used to enumerate live cells by flow cytometry using a FACSDiVa cytometer (BD Biosciences).

**Western blot analysis**

Whole cell lysates from 1.5 x 10⁶ B cells were prepared, separated by electrophoresis, and transferred to membranes as described (20). To verify equivalent protein loading, blots were stripped and reprobed with anti-actin (Santa Cruz Biotechnology). The anti-Blimp-1 mAb was used (18). Primary Abs were revealed using anti-rat-HRP (BD Pharmingen) and anti-goat-HRP (Santa Cruz Biotechnology). For Western blot analyses, B cells were stimulated with 20 µg/ml LPS. The level of Blimp-1 expression in resting B cell populations was measured by comparing the fluorescence of each subset from
Blimp-1<sup>GFP<sup>+/+</sup> mice to the same subset from C57BL/6 control mice (Fig. 1B, solid vs dotted line, respectively). Such direct comparisons were necessitated by each B cell subset having a different level of autofluorescence. Some MZ B cells and B1 cells directly ex vivo showed low but detectable levels of Blimp-1 expression as judged by the shift in GFP fluorescence in comparison to the level of autofluorescence. Some MZ B cells and B1 cells directly ex vivo were necessitated by each B cell subset having a different number in the mouse and the solid line represents cells from a Blimp-1<sup>GFP<sup>+/+<sup> mouse. The number in the top right corner of each FACS profile indicates the median fluorescence value of the Blimp-1<sup>GFP<sup>+/+ population with the background subtracted, as determined using the equivalent nonreporter population. The value, referred to as the δ median fluorescence difference, was calculated from three experiments.

Ig secretion by B1 cell corresponds with Blimp-1 up-regulation

Blimp-1<sup>GFP<sup>+/+<sup> B1 cells from spleen and peritoneum showed negligible Blimp-1 expression (Fig. 1B).

FIGURE 1. Low levels of Blimp-1 can be detected in resting peritoneal B1 and marginal zone B cells. A, FACS gates set for sorting mature B cell subsets. Peritoneal cells were sorted as CD19<sup>+</sup> and B220<sup>−</sup>CD5<sup>+</sup> (peritoneal B2) and B220<sup>−</sup>CD5<sup>+</sup>CD5<sup>−</sup> (peritoneal B1). Spleen cells were sorted as B220<sup>+</sup> and CD21<sup>+</sup>CD23<sup>+</sup> (spleen B2) and CD21<sup>+</sup>CD23<sup>−</sup> (MZ). B, FACS profiles for Blimp-1 expression for each of the mature B cell subsets and ASCs is indicated. The dotted line represents cells from a C57BL/6 mouse and the solid line represents cells from a Blimp-1<sup>GFP<sup>+/+<sup> mouse.

Antibody secreting cell

\[ \text{gfp} \]

\[ \delta \text{median fluorescence} \]

\[ 0.4 \]

\[ 8.1 \]

\[ 3.6 \]

\[ 1.2 \]

\[ 580 \]

FIGURE 2. B1 cells sorted directly ex vivo do not secrete Ig. A, ELISA data for splenic B2 (S B2) and peritoneal B1 (P B1) cells directly ex vivo as compared with ASCs (CD138<sup>GFP<sup>−</sup> ). Data are shown as Ig secreted per 10,000 splenic B2 and peritoneal B1 cells and per 500 CD138<sup>GFP<sup>−</sup> in micrograms per milliliter. The dotted line indicates the threshold for detection in this assay, which is 0.002 μg/ml Ig. B, ELISPOT of peritoneal B1 and control ASCs ex vivo. Over four independent experiments, 10 wells of 10,000 cells each and 10 wells of 1,000 cells each were assayed for Ig secretion from B1 cells, showing no Ig secretion. The result depicted here is for 1,000 B1 cells and 100 ASCs plated per well and assayed for Ig secretion. C, ELISA data for 1,000 cultured B1 cells isolated as GFP<sup>−</sup> (expressing Blimp-1) and 1,000 cells isolated as GFP<sup>+</sup> (not expressing Blimp-1) following stimulation with LPS (20 μg/ml) or CD40L, IL4, and IL5. The dotted line indicates the threshold for detection in this assay of 0.006 μg/ml Ig secreted. D, ELISPOT of GFP<sup>−</sup> cells (Blimp-1 expressing) and GFP<sup>+</sup> cells (not Blimp-1 expressing) sorted from an LPS culture (20 μg/ml) of peritoneal B cells (P B1). Two hundred cells were plated per well. Four independent experiments were conducted assaying over 6,000 cells in total. E, Western blot analysis of Blimp-1 on peritoneal B1 cells with and without LPS stimulation (60 h with 20 μg/ml LPS). The size of each band as determined by a molecular mass marker is displayed. The blot was stripped and reprobed for actin. One of two representative experiments is shown. F, ELISPOT of sorted peritoneal B1 cells (P B1) stimulated for 3 days with LPS (20 μg/ml) from Blimp-1<sup>+/−</sup> or Blimp-1<sup>−/−</sup> cells. These peritoneal B1 cells were sorted from Rag<sup>−/−</sup> or Blimp-1<sup>−/−</sup> embryonic day 12–14 embryos, as the Blimp-1<sup>−/−</sup> mice are embryonic lethal. One thousand cells were plated per well. Two independent experiments were conducted.

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Antibody secreting cell

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these results appear to contradict the finding that a fraction of unstimulated B1 cells secrete Ig without deliberate stimulation (15). This discrepancy may be due to differences in the sensitivity of our assays and/or the means of cell preparation and isolation.

In principle, different subsets of B cells may have different mechanisms or pathways of secretion. It was important, therefore, to investigate whether B1 cells were able to secrete Ig independently of Blimp-1 after stimulation. Accordingly, B1 and B2 cells were partitioned into GFP⁺/H11001 and GFP⁺/H11002 after 72 h of in vitro stimulation with LPS or CD40L, IL-4, and IL-5 and the fractions were assayed for Ab secretion. This showed Ab secretion from B1 cells to be restricted to Blimp-1-expressing cells (Fig. 2, C and D). We confirmed by Western blot analysis that the increase in GFP seen in the stimulated B1 cells correlated with an increase in the Blimp-1 protein (Fig. 2E). The amount of Blimp-1 induced in B1 cells after 60 h stimulation highlights the low levels of the Blimp-1 protein in unstimulated cells (Fig. 2E) and gives an indication of the degree of up-regulation associated with differentiation. Finally, Blimp-1-deficient B1 cells isolated from Rag1⁻/⁻ mice reconstituted with Blimp-1-deficient (Blimp-1 GFP/GFP) fetal liver cells failed to generate ASCs when stimulated with LPS, as measured by ELISPOT (Fig. 2F). These results agree with those obtained using independently derived Blimp-1-deficient B cells (17).

Mature B cell subsets up-regulate Blimp-1 with different kinetics

It has been reported that B1 and MZ B cells can respond rapidly to an Ag (5, 6). We hypothesized that this swift response would involve equally rapid up-regulation of Blimp-1 and the initiation of secretion. Indeed, B1 cells did respond to an Ag rapidly, up-regulating Blimp-1 and initiating Ig secretion within 28 h (Fig. 3A and data not shown). The percentage of ASCs was determined by the quantification of GFP-expressing cells by FACS and confirmed with ELISPOT analysis. The rapid response of B1 cells was not limited to T cell independent-like stimuli of CpG and LPS but extended to CD40L in combination with IL-4 and IL-5 (Fig. 3A). In response to all stimuli, B1 cells up-regulated Blimp-1 more rapidly than MZ B cells and dramatically faster than B2 cells. After 72 h of stimulation with LPS, the percentage of GFP⁺ ASCs was similar for all different B cell types, whereas the other stimuli retained the differential responsiveness seen at earlier times (Fig. 3B). This result was probably not due to different levels of TLR expression among the B cell subsets, because peritoneal B1 and splenic B2 cells express equivalent levels of RP105, TLR2, and TLR4/MD-2 (21). Interestingly, B1 cells showed a response to CpG that B2 cells appeared incapable of emulating; CpG stimulation of B2 cells resulted in very few Blimp-1 positive cells while
B1 cells show an activated transcriptional profile

The rapid up-regulation of Blimp-1 in B1 cells led us to assess the relative levels of several transcription factors known to be involved in ASC differentiation (22). These quantitative RT-PCR samples were first standardized to HPRT and then the comparative expression level of transcription factors in each B cell subset was assessed and presented as a fold difference to the level of splenic B2 cells, which was set at 1. Peritoneal B1 cells had elevated levels of Blimp-1 mRNA and lower levels of both Bcl6 and MTA-3 mRNA as measured by real-time RT-PCR (Fig. 4A). Interestingly, the levels of IRF-4 and XBP-1 mRNA were lower in B1 cells than in other B cell types. These two genes are critical for ASC formation (23) and provide further evidence against B1 cells being able to secrete Ig directly ex vivo. In contrast to a previous report (15), we were able to detect Pax-5 in B1 cells by real-time RT-PCR as well as by intracellular staining and Western blot analysis using a Pax-5-specific Ab (Fig. 4B). The down-regulation of Pax5 in the majority of B1 and a minority of B2 cells following LPS stimulation was clearly visible (Fig. 4B). The expression of Pax-5 in peritoneal B1 cells directly ex vivo has recently been shown by quantitative RT-PCR, as has the subsequent down-regulation of Pax-5 following stimulation (17). The small differences in levels of Pax5 mRNA between each of the B cell subsets revealed by the real-time RT-PCR were not apparent with intracellular staining and Western blot analysis (Fig. 4B), possibly reflecting a degree of posttranscriptional regulation. The quantitative RT-PCR analysis of Blimp-1 expression revealed more Blimp-1 mRNA in both MZ B cells and B1 cells compared with splenic and peritoneal B2 cells, in agreement with the flow cytometry of Blimp-1 reporter B cells (Fig. 1B). Unlike the flow cytometry, however, the quantitative RT-PCR showed more Blimp-1 mRNA in MZ B cells than B1 cells (Fig. 4A). The reason for this discrepancy is unknown but may reflect the level of resolution of the assays.

B1 cells have the potential to home to the bone marrow and form ASCs

The rapid differentiation of B1 cells to ASCs in vitro, with the concomitant up-regulation of Blimp-1, led us to attempt to follow the in vivo differentiation of sorted peritoneal B1 cells into ASCs. It has previously been difficult to track the in vivo differentiation of B cell subsets into ASCs, as the markers available do not detect all ASCs and the surface molecules used to resolve donor and host lymphocytes, such as the Ly5 allosystems, are not expressed on all ASCs. Naive B cells from the Blimp−/− mice and donor ASCs identified by the expression of GFP and CD138. Using CD19−/− mice as recipients allowed the presence of donor B cells to be monitored using CD19 as a marker, while the immunocompromised environment of the CD19−/− mice potentially reduced the competition from endogenous ASCs (24–26).

Purified naive B1 and B2 cells were transferred into CD19−/− recipients with similar efficiency as measured by the recovery of carrier Ly5.1 B cells in the otherwise Ly5.2 recipients (data not shown). The injection of peritoneal Blimp−/− (GFP+) B1 cells into CD19−/− mice resulted in the appearance of ASCs in the spleen and bone marrow as determined at day 6 postinjection (Fig. 5). Following the injection of peritoneal B1 cells, equal numbers of ASCs with high and intermediate (int) levels of GFP were observed in the recipient spleen (GFPint had 442 ± 121.1 and GFPhigh had 448 ± 121.7 ASCs per million recipient spleen cells; n = 3 recipients), while transferred spleen B2 cells predominantly gave rise to ASC with intermediate levels of GFP (GFPint had 296 ± 169.0 and GFPhigh had 50 ± 28.0 ASCs per million recipient spleen cells; n = 3 recipients). The same trend was observed in the bone marrow, both in terms of a higher frequency of ASCs arising
phenotypically more mature than the GFPint subset as judged by the context of biological correlates in vivo; GFPhigh ASCs are longer lived and more resistant to down-regulation in isolated B cell subsets in response to a range of differentiation stimuli that revealed several unique features of the B1 cells response both in vitro and in vivo.

Our results appear to contradict a number of the conclusions in a recent report with respect to the requirements for B1 cell differentiation into ASCs. First, we found that Blimp-1 up-regulation was associated with ASC differentiation in B1 cells as it was in all B cell subsets and in all circumstances examined. That is, we found no-evidence for Blimp-1 independent ASC formation. Second, we found that B1 cells in isolation were not secreting Ig to the extent that was detectable in our assays, either by ELISA or ELISPOT, whereas Tumang et al. (15) found this to be the case. Third, we found B1 cells to contain Pax5 mRNA and protein at levels comparable to that of B2 and MZ B cells, whereas others have failed to detect Pax-5 in B1 cells (15). There are several possible reasons why such differences may have arisen. Two obvious ones are the means of isolating the cells and the sensitivity of the assays used to measure the various parameters. We assessed the secretory capacity of the B cell subsets as soon as possible after their isolation, whereas there appears to have been a period of in vitro culture in the alternate protocol (15) during which a fraction of the B1 cells may have differentiated. The sensitivity of the assays may therefore be moot for Ig secretion but may explain how we detected Pax-5 and Blimp-1 whereas others have not. A further possibility is that the Abs used to identify the cells have in some way altered their subsequent behavior, which may be particularly relevant for CD5 as it is known to have regulatory properties. However, we consider it unlikely that ligation of CD5 on ice would result in the down-regulation of Blimp-1 expression such that the B1 cells would have lost the level necessary for Ig secretion. Collectively, our results show that B1 cells robustly up-regulate Blimp-1 following stimulation and that these stimulated B1 cells are able to secrete quantities of Ig that are similar to sorted, mature ASCs.

The rapid response of peritoneal B1 and MZ B cells to both T-independent and T-dependent stimuli supports the idea that these cells play a crucial part in the early humoral responses to varied forms of Ag (5). The flip side of B1 heightened responsiveness is the possible contribution of B1 cells to autoimmunity. It has been observed that B1 cells produce low-affinity autoreactive but nonpathogenic Abs in wild-type mice. However, in certain strains of manipulated mice and naturally occurring autoimmune prone strains (New Zealand Black and Motheaten Viable), the Ab from B1 cells has been implicated in pathogenicity (29, 30). For example, mice transgenic for the H and L chains of 4C8, an anti-RBC Ig, were found to have cells secreting 4C8 Ab exclusively in the peritoneum, and the frequency of these peritoneal 4C8-secreting cells correlated with disease severity. These 4C8-secreting cells were reported to have the surface phenotype of B1 cells (31, 32), B1 cells have been observed in elevated numbers in patients with the autoimmune diseases Sjögren’s syndrome and rheumatoid arthritis, suggestive that these cells may have some role to play in the pathogenicity of certain autoimmune diseases (33, 34).

What might limit the pathogenicity of autoreactive B cells in a wild-type situation? One possibility is that there is an intrinsic difference in the transcription factors involved in MZ and B1 cell differentiation as opposed to those for B2 cells that results in a unique short-lived ASC. Because Blimp-1 has been hypothesized to play a role in ASC survival (35), we postulated that MZ and B1 cells may be restricted in the level of Blimp-1 that they could

**FIGURE 5.** Differentiation of peritoneal B1 and splenic B2 cells in vivo. A, Five hundred thousand peritoneal (P) B1 and splenic (S) B2 cells were FACS sorted and injected i.v. into CD19-deficient recipients. Six days later the organs were harvested and the presence of ASCs was analyzed on the basis of CD138 staining and GFP expression. These data are representative of three experiments. The polygon indicates those cells judged as plasma cells and the dotted line separates GFPint from GFPhigh cells. An intact Blimp-1GFP+/− mouse is shown for comparison, as is a control CD19+/− mouse into which no reporter cells were injected. The numbers show the percentage of cells for this experiment in each gated region.

from B1 cells and a higher proportion of those being GFPhigh (Fig. 5). Partitioning ASCs on the basis of Blimp-1 levels has a number of biological correlates in vivo; GFPhigh ASCs are longer lived and phenotypically more mature than the GFPint subset as judged by turnover and expression of the various markers that reflect plasma cell maturation, including CD19, MHC II, and CD43 (18). Our observation that B1 cells can give rise to GFPhigh ASCs in the bone marrow is therefore suggestive of their capacity to contribute to the long-lived plasma cell compartment in normal mice. These results also show that the rapid maturation of B1 cells seen in various circumstances in vitro can also occur in vivo.

**Discussion**

It has been proposed that Blimp-1 is the “master” regulator of plasma cell differentiation and, as such, a key molecule in initiating Ab secretion (9). The Blimp-1-deficient mice generated by Shapiro-Shelef et al. (9) in which Blimp-1 was deleted specifically from B cells had drastically reduced serum Ig titers but retained sufficient serum Ig for detection by ELISA. This apparent anomaly could in principle be due to a subset of B cells being able to secrete Ab in a Blimp-1-independent manner. Such a possibility was given support by the report found that B1 cells specifically were indeed able to secrete Ig without any measurable up-regulation of Blimp-1 (15). In this study we demonstrate that B1 cells do not violate the accepted paradigm for the expression of differentiation-associated transcription factors, as has been proposed (15), and are therefore unlikely to be the source of Ig detected in mice with Blimp-1-deficient B cells (9). Other groups have reached this conclusion independently (17). Furthermore, our results showed negligible Ab secretion by B1 cells directly after isolation, as was also the case with splenic B2 cells. Although this has been shown by other groups using different methodologies (27, 28), it differs from other more recent results (15). Finally, we used a flow cytometry reporter system to assess the kinetics and extent of Blimp-1 up-regulation in isolated B cell subsets in response to a range of differentiation stimuli that revealed several unique features of the B1 cells response both in vitro and in vivo.

The rapid response of peritoneal B1 and MZ B cells to both T-independent and T-dependent stimuli supports the idea that these cells play a crucial part in the early humoral responses to varied forms of Ag (5). The flip side of B1 heightened responsiveness is the possible contribution of B1 cells to autoimmunity. It has been observed that B1 cells produce low-affinity autoreactive but nonpathogenic Abs in wild-type mice. However, in certain strains of manipulated mice and naturally occurring autoimmune prone strains (New Zealand Black and Motheaten Viable), the Ab from B1 cells has been implicated in pathogenicity (29, 30). For example, mice transgenic for the H and L chains of 4C8, an anti-RBC Ig, were found to have cells secreting 4C8 Ab exclusively in the peritoneum, and the frequency of these peritoneal 4C8-secreting cells correlated with disease severity. These 4C8-secreting cells were reported to have the surface phenotype of B1 cells (31, 32), B1 cells have been observed in elevated numbers in patients with the autoimmune diseases Sjögren’s syndrome and rheumatoid arthritis, suggestive that these cells may have some role to play in the pathogenicity of certain autoimmune diseases (33, 34).
acquire, thereby limiting their differentiation and subsequent survival as long-lived ASCs. In support of this hypothesis we have recently shown that the expression levels of Blimp-1 are tightly controlled during plasma cell ontogeny (18). However, the levels of Blimp-1 seen in MZ B and B1 cells, once activated, indicate that these cells resemble ASCs generated from follicular cells.

It has recently been shown that Blimp-1 expression levels can be used to divide ASC in vivo into long-lived (GFPhigh) and short-lived (GFPr−) subsets (18). In addition to lifespan, Blimp-1 expression levels in ASC correlate with the changed expression of a panel of markers that are associated with plasma cell maturation (18). The transfer of peritoneal B1 cells resulted in ASCs with both high and intermediate levels of GFP, suggesting that B1 cells can contribute to the mature, long-lived ASC compartment. Indeed, the B1 cell-derived plasma cells were indistinguishable from the ASCs that differentiated from their B2 cell counterparts in terms of CD138 and Blimp-1 expression and anatomical location. A potential limitation of this experiment is that it was conducted in the absence of a competent immune system, thus reducing the potential competition between ASCs for niches within the bone marrow (26). It does show, however, that B1 cells have the potential to become ASCs in the bone marrow.

There are several transcription factors, including Blimp-1, Pax-5, Bcl6, and MTA3 that play important roles in the differentiation of B2 cells (23). Because MZ and B1 cells are proposed to arise as a result of a different maturational program than that of B2 cells (36, 37), it was plausible that transcription factors that orchestrate B2 cell differentiation would be irrelevant for B1 or MZ cell differentiation. However, our analysis of the expression pattern of Blimp-1 and Pax-5 in differentiating B1 and B2 cells indicated that both cell types follow broadly similar transcriptional control programs. Interestingly, B1 cells analyzed directly ex vivo expressed increased levels of Blimp-1 and reduced Pax-5, Bcl6, and MTA3 compared with B2 cells, suggesting that these cells are predisposed to differentiate into ASCs. This transcriptional priming would explain the rapid differentiation of B1 cells into ASCs in vitro and raises the possibility that they require fewer additional signals to trigger ASC differentiation than B2 cells. MZ cells also show elevated levels of Blimp-1 and decreased levels of Bcl6, indicating that it may be the level of these two genes that determine the kinetics of the response of B cells to stimuli. In particular the transcription level of Bcl6 in MZ and B1 cells is interesting. Bcl6 has been shown to directly repress cyclin D2, a G1 cell cycle regulator (38), and it has been shown that B1 cells rapidly induce cyclin D2 in response to PMA stimulation (39). It may be that the release of Bcl6 repression of cyclin D2 in MZ and B1 cells results in a faster cell cycle initiation in these cells once they receive stimulation.

Xbp-1 is a gene that has been shown to play a role in endoplasmic reticulum expansion (40) and is an essential component of the transition from a B cell to an ASC (10). The quantitative RT-PCR for this gene indicated that in MZ and B1 cells, which are able to rapidly become ASCs, the level of xbp-1 is not elevated. This supports recently published results for MZ and B2 cells (6) showing that transcripts encoding several ER resident proteins important for protein folding and secretion were elevated in resting MZ B cells compared with resting splenic B2 B cells despite similarities in xbp-1 expression. These results, in combination with the other results described from these quantitative RT-PCRs, indicate that B1 cells show a transcriptional priming that predisposes these cells toward a rapid response to stimulation.

It was not only the kinetics of B1 and MZ cell differentiation that were of interest but the response of B1 cells to CpG. Although CpG stimulated all subsets of B cells to proliferate, B1 cells were unique in their substantial up-regulation of Blimp-1. It has recently been shown that chromatin-IgG complexes are able to signal through TLR9 and activate autoreactive B cells that would otherwise typically remain quiescent (41). The response of B1 cells to CpG indicates that there is an inherent difference in the response of B1 and B2 cells to this stimulus and suggests that B1 cells should be investigated to ascertain whether they are subject to the same B cell tolerance mechanisms that suppress autoantibody secretion by B2 cells in response to this stimulus (42).

As we search for therapies to various autoimmune diseases in which B cells play a role, the potential of B1 cells, which may be of an inherently self-reactive nature (1), to make long-lived Abs poses interesting and important considerations for therapy design. The system described here using Blimp-1 reporter B cells will be useful in exploring the nature of the longevity and pathogenicity of ASCs that result from B1 cells.

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