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Plasma Cell Ontogeny Defined by Quantitative Changes in Blimp-1 Expression

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Abstract

Plasma cells comprise a population of terminally differentiated B cells that are dependent on the transcriptional regulator B lymphocyte–induced maturation protein 1 (Blimp-1) for their development. We have introduced a *gfp* reporter into the *Blimp-1* locus and shown that heterozygous mice express the green fluorescent protein in all antibody-secreting cells (ASCs) in vivo and in vitro. In vitro, these cells display considerable heterogeneity in surface phenotype, immunoglobulin secretion rate, and *Blimp-1* expression levels. Importantly, analysis of in vivo ASCs induced by immunization reveals a developmental pathway in which increasing levels of *Blimp-1* expression define developmental stages of plasma cell differentiation that have many phenotypic and molecular correlates. Thus, maturation from transient plasmablast to long-lived ASCs in bone marrow is predicated on quantitative increases in *Blimp-1* expression.

Key words: Prdm1 • B-lymphopoiesis • plasma cell • antibody secretion • terminal differentiation • syndecan-1

Introduction

Plasma cells are the end point of B cell lineage differentiation and are essential for protective immunity. The short-lived antibody-secreting cells (ASCs) arise in extrafollicular sites in response to primary immunization, persist for only a few days, and produce antibody of relatively low affinity. In contrast, long-lived ASCs produced in the T-dependent germinal center pathway undergo affinity maturation and reside primarily in the BM (1). Long-lived ASCs are maintained independently of antigen by intrinsic longevity, as well as being replenished by the differentiation of memory B cells (2). Despite several decades of research, the regulation of plasma cell development is poorly understood. Although there is general agreement that three stages of plasma cells can be identified (plasmablast, short-lived, and long-lived plasma cells), the developmental relationship between them is unclear as are the factors that may mediate such maturation.

The B lymphocyte–induced maturation protein 1 (Blimp-1/Prdm1) has been proposed to have a preeminent role in regulating B cell terminal differentiation for the following reasons. *Blimp-1* is expressed in ASCs from human and mouse, but not in memory cells (3). Notably, ectopic expression of *Blimp-1* is sufficient to drive differentiation to a ASC phenotype (4–7). Antisense approaches (8) or a dominant-interfering Blimp-1 (9) are able to suppress exit from the cell cycle, a change essential for full ASC differentiation. In line with these studies, it has been recently demonstrated that mice lacking Blimp-1 in B cells produce greatly decreased levels of Ig and have a markedly reduced ASC compartment (10). Clearly, *Blimp-1* expression is a key determinant in plasma cell development.

Blimp-1 is a transcriptional repressor that binds to DNA via conserved zinc finger motifs (11) and can interact with corepressors such as Groucho, histone deacetylases (12, 13), and the histone H3 methyltransferase, G9a (14). Blimp-1 repression is postulated to be essential for the extinction of *c-myc* expression and the exit from the cell cycle characteristic of terminal differentiation (15, 16). Blimp-1 directly represses the promoter of the *Pax5* gene (17). Pax5 is required for the maintenance of B cell identity and represses the expression of *XBP-1*, itself an essential player in plasma cell development (18, 19). Many other putative Blimp-1 repressed genes have been identified using microarray technology; however, most have not been validated in the absence

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Abbreviations used in this paper: ASC, antibody-secreting cell; Blimp-1, B lymphocyte–induced maturation protein 1; BrdU, bromodeoxyuridine; ES, embryonic stem; IRES, internal ribosome entry site; KLH, keyhole limpet hemocyanin; NP, 4(hydoxy-3)-nitrophenyl acetyl; Synd-1, syndecan-1.

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of *Blimp-1* expression (20, 21). Collectively, these data support the notion of Blimp-1 expression being a master regulator of plasma cell differentiation.

The study of plasma cells is hampered by their heterogeneity in lifespan, surface phenotype, location, and the absence of virtually all B lineage–associated markers, making identification and isolation of ASCs a limiting step in their characterization (1, 22–25). To overcome this difficulty, we have generated a mouse model where *gfp* has been introduced into the *Blimp-1* locus. We show that *Blimp*^{gfp} provides, for the first time, a definitive methodology to identify all plasma cells and reveals significant phenotypic heterogeneity in the ASC compartment. Moreover, the regulated expression of *Blimp*^{gfp} defines the ontogeny of B cells from plasmablasts to long-lived plasma cells.

Materials and Methods

Generation of the Blimp^{gfp} *Mice.* The pKW11 vector (obtained from M. Busslinger, IMP, Vienna, Austria) consisting of a splice acceptor, stop codons in all reading frames, an internal ribosome entry site (IRES), *eGFP* cDNA, and a SV40 polyadenylation signal. The *PGK-Neo*^{r} gene allowed for the selection of embryonic stem (ES) cells with an integrated targeting vector. Genomic DNA sequences adjacent to exon 6 (5′ 4 kb and 3′ 3 kb) were amplified from a *Blimp-1*–containing BAC and cloned as homology arms into pKW11 to produce the targeting vector. C57BL/6 ES cells were electroporated with linearized targeting vector, and resistant clones were selected and screened by Southern hybridization to 5′ and 3′ genomic DNA probes. Four targeted clones were injected into BALB/c blastocysts to obtain chimeric founders. Germline transmission has been achieved with two clones. Mice were bred and maintained at the Walter and Eliza Hall Institute under Animal Ethics committee guidelines.

Genotyping. Genomic DNA was digested with SpeI (3′ arm) and hybridized to a 500-bp fragment of PCR-amplified genomic DNA 3′ to the homology arms (see Fig. 1 A). C57BL/6 DNA gave a band of 5.8 kb, whereas correctly targeted clones gave an additional 4.5-kb band. PCR genotyping was performed using the primer combination: bl-1 5′-GGCAAGATCAAGTATGAGTGC-3′, bl-2 5′-TGAGTAGTCACAGAGTACCCA-3′, and bl-3 5′-GCGGAATTCATTTAATCACCCA-3′. PCR fragments of 611 and 531 bp were indicative of wild-type and targeted alleles, respectively.

Transplantation of Fetal Liver Cells. *Blimp*$^{gfp/+}$ (C57BL/6 Ly5.2) mice were intercrossed; fetal liver cells were isolated from E14.5 embryos and genotyped by PCR; and 1–3 × 10^6 fetal liver cells were injected into lethally irradiated C57BL/6 *Rag1*$^{-/-}$ Ly5.1 congenic recipients (2 × 550 rad). Mice were analyzed after 8 wk. Successful reconstitution was analyzed using a Ly5.2-specific mAb.

Flow Cytometry. The mAbs against CD19 (1D3), B220 (RA3-6B2), and Ly5.2 (ALI-4A2) were purified from hybridoma supernatants on protein G–Sepharose columns (Amersham Biosciences) and conjugated to biotin (Pierce Chemical Co.), allophycocyanin, phycoerythrin (ProZyme), and Alexa Fluor 633 (Molecular Probes) as recommended by the suppliers. Anti–syndecan-1 (Synd-1; 281-2), MHCII (M5/114.15.2), CD43 (S7), CD62L (MEL-14), CD38 (90), CXCR4 (2B11), and CXCR5 (2G8) were obtained from BD Biosciences. Cells were analyzed on an LSR cytometer (BD Biosciences), and cell sorting was performed on high-speed flow cytometers (Moflo; DakoCytomation and BD Biosciences). In vivo cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation. Mice were given an i.p. injection of 0.2 mg BrdU in PBS on day 1 and placed on BrdU drinking water (0.5 mg/ml in 2% glucose) for 4 d. GFP$^+$ cell populations were sorted and fixed before being analyzed for BrdU uptake using an allophycocyanin-conjugated anti-BrdU mAb (BD Biosciences), following the protocol supplied by the manufacturer.

ELISA and ELISPOT Assay. Ig levels were measured using ELISA as described previously (29). Antibodies were purchased from Southern Biotechnology Associates, Inc. and streptavidin–horseradish peroxidase or streptavidin–AP conjugates were obtained from Sigma-Aldrich. ELISPOT assays were performed on MultiScreen-HA filter plates (Millipore). Cells were incubated for 4 h at 37°C on precoated 96-well filter plates and developed with AP substrate. Experiments were performed three times in triplicate.

In Vivo Induction of ASCs. 2 μg *Escherichia coli* LPS (Sigma-Aldrich) was injected intravenously into *Blimp*$^{gfp/+}$ mice, and animals were analyzed daily for up to 7 d. Immunization was with a single i.p. injection of 100 μg 4(hydroxy-3)-nitrophenyl acetyl (NP) coupled to keyhole limpet hemocyanin (KLH) in the ratio of 13:1 (26). The antigen was precipitated onto alum and washed extensively before injection. Single cell suspensions from spleen and BM were analyzed as described previously (26). IgG1 ASC activity in 500 sorted GFP$^+$ populations was determined using NP-specific ELISPOT (26).

In Vitro Cell Culture. Naive B cells were purified from spleens by T cell complement depletion, Percoll gradient centrifugation, and B220 magnetic bead purification (Miltenyi Biotec) as described previously (29). Purified cells (95% IgM$^+$IgD$^+$B220$^+$) were cultured at 10^5/ml with optimal concentrations of CD40L, 500 U/ml IL-4, and 2 ng/ml IL-5. 4 × 10^5 cells/ml were used for 20 μg/ml LPS stimulation. Cell membranes expressing mouse CD40L were prepared from Sf21 cells (42). Recombinant mouse IL-4 was obtained from R. Kastelein (DNAX Research Institute, Palo Alto, CA), and IL-5 was purchased from R&D Systems. Cell proliferation was assessed by pulsing cultures for 2 h with 1 μCi [methyl-^{3}[H]thymidine (Amersham Biosciences). Cells were harvested onto glass-fiber mats, and incorporation was determined by scintillation counting.

Western Blotting. An anti–Blimp-1 mAb was generated in rats by immunization with a purified GST–Blimp-1 fusion protein consisting of the 141 amino acids lying between the PR and proline-rich domains of mouse Blimp-1 fused to the COOH terminus of glutathione-*S*-transferase in the vector pGEX-KT. mAbs were screened by Western blotting using B cell lines representing B cell and plasma cell stages. Reactivity with an endogenous protein of the appropriate size for Blimp-1 (~95 kD) protein was specifically detected for clone 6D3. Total protein extracts were produced from equivalent numbers of cells, and Western blotting was performed as described previously (43). Equal protein loading was confirmed using goat anti-ICSBP (C-19) and β-actin (I-19) obtained from Santa Cruz Biotechnology, Inc.

RT-PCR Analysis. In vitro–cultured B cells were sorted and subjected to RT-PCR as described previously (44). PCR products were separated on agarose gels and visualized by ethidium bromide staining. Primer sequences are available upon request.

Results

Generation of a Blimp^{gfp} *Reporter Allele.* Gene targeting of the *Blimp-1* locus resulted in the insertion of an *IRES-GFP* cassette 3′ to exon 6 to produce the *Blimp*^{gfp} allele

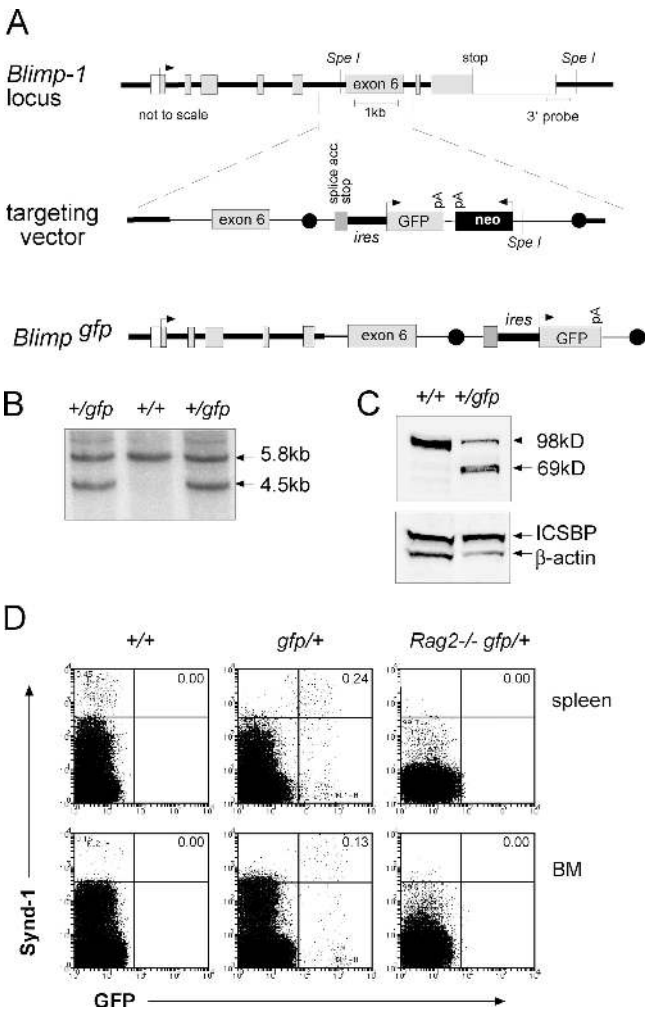


Figure 1. Generation of Blimp^{gfp} reporter mice. (A) The genomic locus of *Blimp-1*, indicating the exons as boxes and introns as black lines. Coding regions are in gray and nontranslated regions are white. SpeI sites used for Southern hybridization along with the 3′ probe are marked. Arrows indicate direction of translation from initial methionine. The targeted allele derived from the homologous recombination event is indicated. pA, polyadenylation signal sequence; circles, frt sites; triangles, stop codon; splice acc., splice acceptor. The targeted allele encodes a truncated Blimp-1 protein (Blimp^{trunc}) lacking exons 7–8 and GFP from the same mRNA transcript. (B) Southern hybridization of SpeI digested *Blimp$^{gfp/+}$* and C57BL/6 ES cell DNA. (C) Western blot analysis of wild-type and *Blimp$^{gfp/+}$* splenic B cells cultured for 4 d in LPS. The wild-type and Blimp^{trunc} proteins are indicated. β-actin and ICSBP-specific antibodies were used as loading controls. (D) Flow cytometric analysis of spleen and BM. A small population of GFP$^+$ cells was present specifically in the *Blimp$^{gfp/+}$* mice and lost in wild-type or *Rag2$^{-/-}$Blimp$^{gfp/+}$* mice.

(Fig. 1 A). Homologous recombination in a C57BL/6-derived ES cell line was confirmed by Southern hybridization using flanking sequences (Fig. 1 B). This strategy resulted in a truncated Blimp-1 protein (Blimp^{trunc}) that lacked the zinc finger motif domains encoded by exons 7–8 (Fig. 1 C). Most importantly, the targeting strategy also produced a *Blimp-1* reporter allele as the GFP was expressed from the bicistronic *Blimp-IRES-GFP* mRNA under the control of endogenous *Blimp-1* regulatory elements.

Heterozygous *Blimp$^{gfp/+}$* mice developed normally and were indistinguishable from C57BL/6 mice in terms of lymphoid cellularity, B cell differentiation, and serum Ig titers (unpublished data). In contrast, *Blimp$^{gfp/gfp}$* embryos die in late gestation. However, Blimp-1–deficient hematopoiesis could be examined by fetal liver reconstitution of lethally irradiated *Rag1$^{-/-}$ Ly5.1* recipients. The grossly normal reconstitution of lymphoid and myeloid lineages in these chimeras indicated that Blimp-1 was not essential for stem cell self-renewal or hematopoiesis in general (unpublished data).

Analysis of lymphoid organs revealed that the vast majority of cells expressed no GFP, whereas a minority expressed detectable but low levels (Fig. 1 D). In contrast, high level Blimp^{gfp} expression was restricted to a rare fraction of cells in lymphoid tissues (from 0.1 to 0.5%), many of which also expressed Synd-1, a commonly used marker of ASCs. High level GFP fluorescence was absent from wild-type or lymphoid-deficient *Rag2$^{-/-}$Blimp$^{gfp/+}$* cells (Fig. 1 D). *Blimp$^{gfp/gfp}$* reconstituted animals lacked a distinct GFP^{hi} compartment and Synd-1 expression (unpublished data). Consistent with a previously published paper (10), *Blimp$^{gfp/gfp}$* reconstituted mice had severely reduced numbers of BM and splenic plasma cells as measured by ELISPOT (unpublished data).

All Blimp^{gfp} High Cells Are ASCs. To determine the concordance between high *Blimp^{gfp}* expression in the heterozygous reporter mice and ASC function, we performed Ig ELISPOT assays on sorted cell populations from spleen and BM using GFP as the only sorting parameter. These experiments showed that *Blimp^{gfp}*-expressing cells represented a pure population of ASCs, as the GFP$^+$ fraction contained a high proportion of Ig-secreting cells, whereas 10^5 GFP$^-$ cells lacked ASC activity (Fig. 2 A). The GFP$^+$ population contained all Ig isotypes at the expected ratios and, furthermore, the proportion of ASCs was similarly high in both the Synd-1$^+$GFP$^+$ and Synd-1$^-$GFP$^+$ fraction (Fig. 2 A). Therefore, the *Blimp^{gfp}* reporter allele allows the single parameter identification of all ASC, with an enrichment of ~10^5-fold over nonexpressing cells.

Plasma Cells Are Functionally Heterogeneous. Although all GFP$^+$ cells were ASCs, it was apparent that there was heterogeneity in the Blimp^{gfp} fluorescence levels in lymphoid organs. Splenic ASCs were either GFP-intermediate (GFP^{int}) or GFP^{hi}, whereas the BM ASCs were even higher for GFP fluorescence. The heterogeneous *Blimp-1* expression was also apparent at the mRNA level (Fig. 2 D). These results suggested a differentiation process visualized by increased *Blimp-1* expression (Figs. 1 D and 2 B), a concept supported by the progressive loss of B cell markers (CD19, B220, and MHCII) from spleen GFP^{int} compared with BM GFP^{hi} ASCs (Fig. 2 C).

The ability to identify distinct populations of ASCs based on *Blimp-1* expression levels enabled us to examine their cell surface phenotype. Synd-1 expression is commonly used to identify mouse ASCs, although there are reports of Synd-1$^-$ ASCs (22). Analysis of *Blimp^{gfp}*-expressing cells revealed the existence of Synd-1$^+$ and Synd-1$^-$ ASC, with

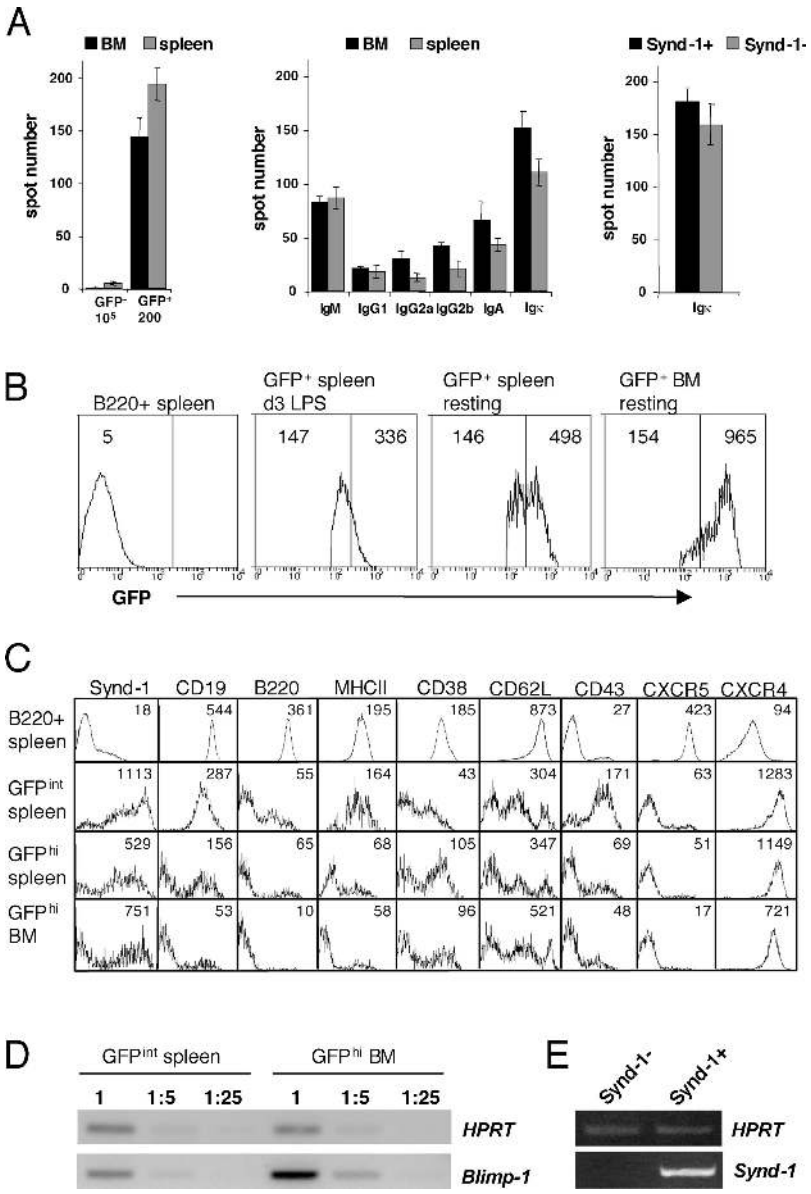


Figure 2. All GFP$^+$ cells are ASC. (A, left) 10^5 GFP$^-$ or 200 GFP$^+$ cells from BM or spleen of *Blimp*$^{gfp/+}$ mice were assayed for Igκ secretion by ELISPOT. Middle panel, GFP$^+$ cells were sorted from the BM and spleen of *Blimp*$^{gfp/+}$ mice and subjected to ELISPOT assay for the indicated isotype. (right) GFP$^+$Synd-1$^+$ and GFP$^+$Synd-1$^-$ cells were sorted from BM of *Blimp*$^{gfp/+}$ mice assayed for Igκ secretion. Mean number of ELISPOT per 200 cells ±SE is shown. (B) Gated populations show the level of GFP in splenic B220$^+$ B cells and GFP$^+$ ASC from spleen (LPS induced and resting) and BM. GFP$^+$ cells left of the dividing line were considered GFP intermediate (GFP^{int}) and those to the right were considered GFP high (GFP^{hi}). (C) Analysis of the surface phenotype of gated cells from B. Mean fluorescence index is indicated for each histogram. (D) Sorted GFP^{int} and GFP^{hi} cells were sorted as for B and subjected to semi-quantitative RT-PCR analysis. Serial fivefold dilutions of the cDNA were analyzed. (E) BM GFP$^+$Synd-1$^+$ and GFP$^+$Synd-1$^-$ cells were sorted and assayed for *Synd-1* mRNA. *HPRT* was a loading control.

the majority of Synd-1$^-$ cells being GFP^{hi} (Figs. 1 D and 2 C). RT-PCR analysis confirmed that the loss of *Synd-1* expression occurred at the transcriptional level and was not the result of shedding (Fig. 2 E). GFP$^+$ cells were also heterogeneous for other reported ASC markers examined, including CD43, CD62L, and CD38 (Fig. 2 C). In contrast, the chemokine receptors CXCR5 and CXCR4 were modulated as expected for an ASC population (Fig. 2 C). Thus, plasma cells are a heterogeneous population defined by increasing *Blimp-1* expression.

Induction of Blimp-1 Expression by Polyclonal and Antigen-specific Stimuli. Antibody secretion and *Blimp-1* expression are induced by antigen-specific and polyclonal stimuli (3–5, 8). We have used LPS to examine the kinetics and phenotype of ASCs induced in vivo. LPS injection increased the numbers of GFP-expressing cells in the spleen from the resting levels of 0.6 ± 0.2% to a peak of 4.7 ± 1.9% after 3 d (Fig. 3 A). Induced cells subsequently appeared in the BM at day 4 and were ASCs as determined by ELISPOT assay (Fig. 3 B and not depicted). The numbers of GFP$^+$ ASCs in both locations rapidly declined, returning to resting values by day 7. Analysis of the surface phenotype of the splenic ASCs suggested that induced *Blimp*^{gfp} expression occurred in GFP^{int}B220$^+$Synd-1$^+$ cells that subsequently lost B220 and acquired a heterogeneous phenotype for Synd-1 (Fig. 3 A). A very similar profile was observed in the BM. As expected, a similar time course with *Blimp*$^{gfp/gfp}$ mice revealed no induction of GFP$^+$ cells or increase in serum IgM (unpublished data).

Next, we examined the development of ASCs in response to a T cell–dependent antigen. Mice were immunized intraperitoneally with the hapten NP coupled to the protein carrier (NP-KLH; reference 26). At regular intervals, spleen and BM were examined for the frequency of

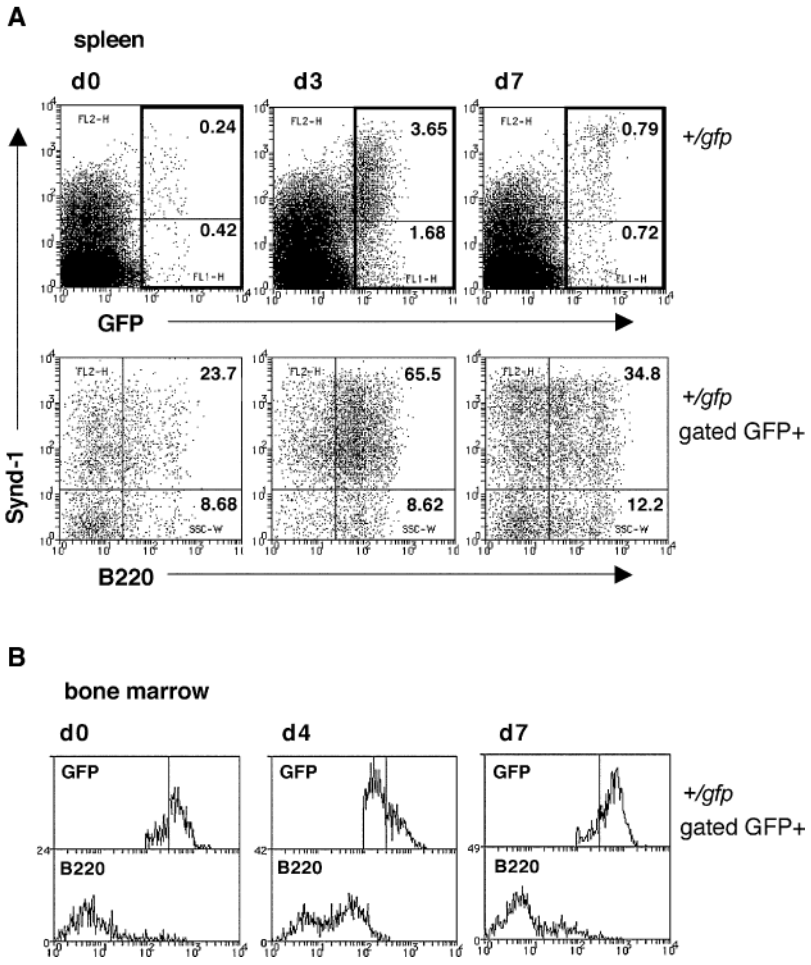


Figure 3. In vivo induction of GFP+ ASC. (A) *Blimp*$^{gfp/+}$ mice were injected with 2 μg LPS and analyzed for Synd-1, GFP, and B220 at the indicated time points. (top) Total splenic cells. (bottom) Gated GFP+ cells only. Gated regions are outlined by thick lines. Percentages of GFP+ cells are indicated. (B) Gated GFP+ cells from the same mice were analyzed for relative GFP fluorescence and B220 expression.

GFP+ cells and the presence of NP-specific ASCs. As expected, immunization resulted in a rapid and significant increase in the proportion of GFP+ cells in the spleen, reaching a peak at day 7 and declining to near resting levels by day 14 (Fig. 4, A and B). This matches the rise and fall in the frequency of antigen-specific ASCs as measured by ELISPOT (27) or histology (28). Interestingly, when the GFP+ population was fractionated into GFP^{int} and GFP^{hi}, it was apparent that there was a rapid increase in the GFP^{int} population in the context of a relatively stable GFP^{hi} population (Fig. 4 C). Comparison of these populations revealed a high frequency of NP-specific IgG1 ASCs

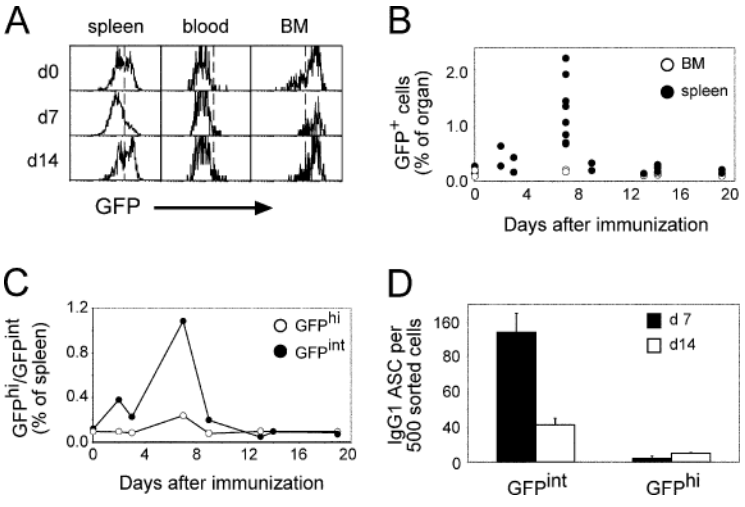


Figure 4. Development of distinct ASC populations after T cell–dependent immunization. (A) Fluorescence levels of GFP+ cells in spleen, blood, and BM from representative *Blimp*$^{gfp/+}$ mice at the indicated times after a single i.p. immunization with NP-KLH in alum. (B) Percentage of GFP+ spleen and BM cells at the indicated time points after immunization. Each circle is an individual mouse. (C) Kinetics of the appearance of GFP^{int} and GFP^{hi} populations in spleen. (D) Frequency of anti-NP IgG1 ASC in GFP^{int} and GFP^{hi} populations in spleen at day 7 (shaded) and day 14 (unshaded) after immunization. Data are from sorted GFP+ cells from three individuals at day 7 and two at day 14.

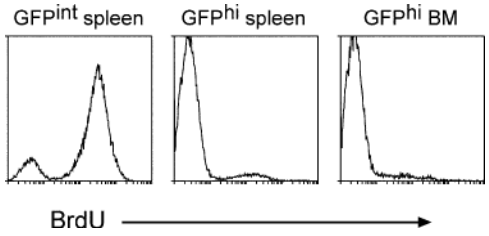


Figure 5. GFP^{int} cells are short-lived plasmablasts. *Blimp*$^{gfp/+}$ mice were given a bolus of BrdU and fed BrdU in the drinking water for 4 d. Cells were sorted for the indicated GFP levels from spleen and BM and fixed, and the incorporation of BrdU for each sample was determined by flow cytometry using a BrdU-specific antibody. Data are from two pooled individuals and are representative of three experiments.

in the GFP^{int} population, compared with the GFP^{hi} population, although the frequency in the GFP^{hi} compartment increased over time (Fig. 4 D). These data suggest that the GFP^{int} population is the rapidly expanding plasmablast population localized in the splenic foci, whereas the GFP^{hi} ASCs appear to be the more stable, long-lived ASC compartment of the spleen. The frequency of Synd-1^{+}GFP^{+} cells in blood changed little as a result of immunization, averaging 0.043 ± 0.026% (n = 19) over the 2-wk period. Interestingly, although the ASC compartment of the BM was exclusively GFP^{hi}, we could only detect GFP^{int} cells in the blood (Fig. 4 A). These results agreed with the appearance of GFP^{int} ASCs in the BM after LPS injection and suggest that the up-regulation of *Blimp-1* expression in the long-lived plasma cell compartment occurred after entry to the BM.

To test our hypothesis that the GFP^{int} cells were short-lived, unimmunized mice were fed BrdU in the drinking water for 4 d. Flow cytometric analysis confirmed that the majority of GFP^{int} cells had turned over, whereas very few GFP^{hi} cells in either spleen or BM were cycling (Fig. 5). It is interesting to note that the frequency of GFP^{+} cells in BM did not change as a result of immunization, even though the frequency of cycling splenic GFP^{int} and GFP^{hi} cells increased markedly (Fig. 4, A and B, and not depicted). In summary, the induction of GFP^{int} population by both polyclonal and antigen-specific immunization, in the context of a relatively stable population of long-lived GFP^{hi} cells, suggests a plasma cell maturation pathway reflected by levels of *Blimp-1* expression and that the GFP^{int} cells are plasmablasts.

Blimp^{gfp} *Allows the Tracking of Plasma Cell Differentiation In Vitro.* The exquisite specificity of Blimp^{gfp} as a marker for ASCs in vivo suggested that the reporter would also be an indicator of ASC differentiation in vitro. Purified small

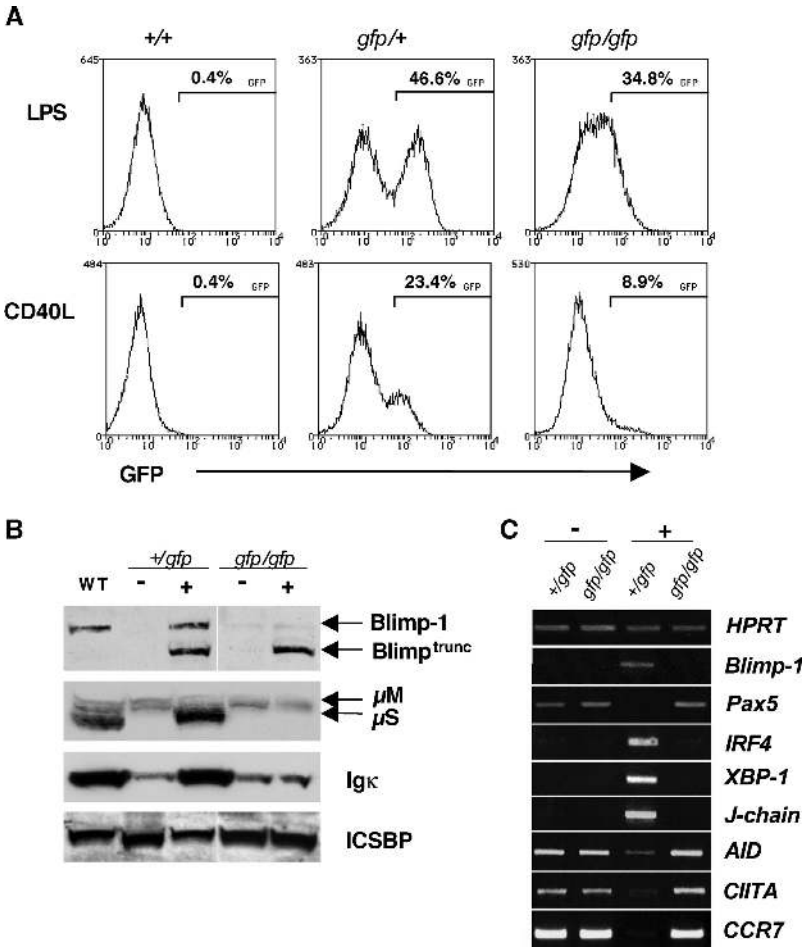


Figure 6. Induction of ASC differentiation in vitro requires Blimp-1. (A) Wild-type, *Blimp*$^{gfp/+}$, and *Blimp*$^{gfp/gfp}$ B cells were cultured in the presence of LPS or the combination CD40L/IL-4/IL-5 for 4 d and examined for GFP expression. Percentages of GFP^{+} cells are shown. (B) Western blot analysis of splenic B cells cultured in LPS and sorted according to GFP expression. Wild-type cells (WT) are unsorted; (+) GFP^{+}; (−) GFP^{-}. The Blimp-1 proteins and the membrane-bound (μM) and secreted IgM (μS) are indicated. Detection of ICSBP was a loading control. White lines indicate intervening lanes have been spliced out. (C) Cells sorted as in B were subjected to RT-PCR analysis for the indicated genes. *Blimp-1* primers span exons 7–8 (not expressed from the targeted allele). *Blimp*$^{gfp/gfp}$ cells do not initiate the ASC transcriptional cascade and remain indistinguishable from the GFP^{-} cells. *HPRT* was used to normalize the relative cDNA input.

resting splenic B cells were cultured with conditions that mimic T cell help (CD40L/IL-4/IL-5) or microbial stimuli (LPS) for 4 d and analyzed for ASC phenotype, class switching, and Ig production (29). As expected, the *Blimp*$^{gfp/+}$ cultures contained a population of GFP$^+$ cells that were absent in the wild-type cultures (Fig. 6 A). Notably, *Blimp*$^{gfp/gfp}$ B cells did express some GFP, indicating that the initial stages of the ASC pathway were induced; however, no cells expressing high levels of GFP or Synd-1 were formed (Fig. 6 A and not depicted). Moreover, *Blimp*$^{gfp/gfp}$ B cells, although normally capable of proliferating and class switching in response to exogenous stimuli, secreted little antibody compared with *Blimp*$^{gfp/+}$ or wild-type cultures (unpublished data). Western blotting of sorted GFP$^+$ cells from *Blimp*$^{gfp/+}$ cultures confirmed that Blimp-1 expression correlated with high levels of Igκ and the secreted form of IgH (μS) as compared with GFP$^-$ cells from the same cultures. In contrast, although Blimp^{trunc} expression was observed in deficient cells, they failed to up-regulate either Ig chain (Fig. 6 B).

Blimp-1 Is Required for the Induction of the Plasma Cell Transcriptional Program. Blimp-1 has been shown to repress transcription of several genes associated with the mature B cell phenotype (21, 30). The Blimp^{gfp} reporter enabled us for the first time to isolate and characterize gene expression in a purified population expressing endogenous Blimp-1. Cells were sorted on the basis of GFP expression and subjected to Western blotting and RT-PCR. Analysis of *Blimp-1* mRNA levels confirmed the coincidence of *Blimp-1* and *gfp* expression in *Blimp*$^{gfp/+}$ cells and the lack of *Blimp-1* exons 7–8 transcripts in *Blimp*$^{gfp/gfp}$ cells (Fig. 6 C). Interestingly, the expression of *Pax5* and several putative Pax5 target genes, including *XBP-1* (18), *J-chain* (31), *AID* (32), and *CIITA* (33), were deregulated in mutant cells. (Fig. 6 C). In addition, *IRF4*, an essential transcriptional regulator of ASC function, was not induced in *Blimp*$^{gfp/gfp}$ cells. Several chemokine receptors are differentially expressed between mature B cells and ASCs, including CXCR5 and CXCR4 (Fig. 2 C and reference 34). We have examined by RT-PCR a panel of other receptors, including CCR7 and

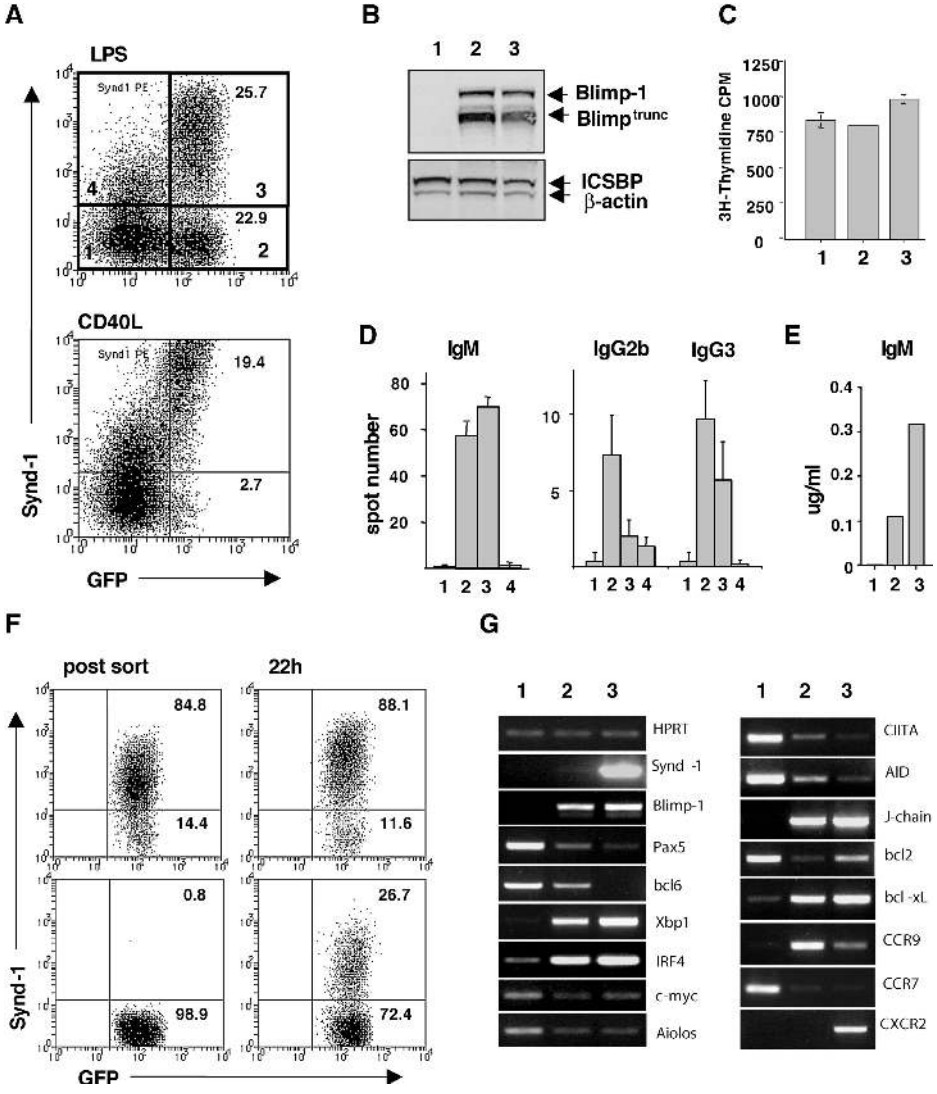


Figure 7. *Blimp*^{gfp} identifies a heterogeneous population of ASC in vitro. (A) *Blimp*$^{gfp/+}$ B cells were stimulated with LPS or CD40L/IL-4/IL-5 for 4 d and examined for Synd-1 and GFP. Four populations GFP$^-$Synd-1$^-$ (fraction 1), GFP$^+$Synd-1$^-$ (fraction 2), GFP$^+$Synd-1$^+$ (fraction 3), and GFP$^-$Synd-1$^+$ (fraction 4) cells were sorted and assayed as follows. (B) Western blotting for Blimp-1 protein. ICSBP and β-actin were used as loading controls. (C) Cell proliferation rates. Sorted cells were pulsed with ^{3}[H]thymidine for 2 h. (D) Ig secretion by ELISPOT. Mean numbers of ELISPOT per 100 cells are shown ±SE. (E) IgM secretion. Sorted cells were recultured for 4 h, and supernatants were analyzed by ELISA. (F) Fractions 2 (GFP$^+$/Synd-1$^+$) and 3 (GFP$^+$/Synd-1$^-$) were sorted (left) and recultured for 22 h in LPS before reanalysis (right). (G) RT-PCR analysis of the indicated genes was performed on the sorted groups. *HPRT* was used to normalize the relative cDNA input.

CCR9, neither of which were regulated normally in the absence of Blimp-1 (Fig. 6 C and not depicted).

Heterogeneity of ASCs In Vitro. Having observed the heterogeneity of plasma cells in vivo, we were interested to see if this diversity also occurred in vitro. Examination of the LPS-stimulated in vitro differentiation of *Blimp*$^{gfp/+}$ B cells revealed this was indeed the case, as only ~50% of the GFP$^+$ cells were Synd-1$^+$. In contrast, most (>85%) GFP$^+$ cells were Synd-1$^+$ in CD40L/IL-4/IL-5 stimulated cultures, demonstrating that this ASC heterogeneity can be modified by extrinsic signals (Fig. 7 A).

To examine more closely this heterogeneity, we sorted GFP$^-$Synd-1$^-$ (fraction 1), GFP$^+$Synd-1$^-$ (2), GFP$^+$Synd-1$^+$ (3), and GFP$^-$Synd-1$^+$ (4) cells from LPS cultures (Fig. 7 A). As expected, GFP exactly coincided with Blimp-1 protein (Fig. 7 B), and ASC activity was restricted to the GFP$^+$ fractions (Fig. 7 D). Interestingly, the rate of IgM secretion was reproducibly lower in the Synd-1$^-$ fraction2 compared with those cells expressing Synd-1 (Fig. 7 E). It has been reported that *Blimp-1*$^{-/-}$ B cells are hyperproliferative in response to LPS (10); however, determination of the cell proliferation rate of the sorted fractions revealed no relationship between Blimp-1 expression and cell proliferation (Fig. 7 C). The negative effect of Blimp-1 on proliferation is reported to require the repression of *c-myc* (16, 35). RT-PCR analysis of the three fractions indicated that *c-myc* was only slightly down-regulated in the presence of Blimp-1, arguing against an important role of this process in ASCs in vitro (Fig. 7 G).

The phenotypic diversity in the sorted fractions was further assayed by RT-PCR. Both GFP$^+$ populations displayed the hallmarks of ASC differentiation (decreased *Pax5*, *AID, CIITA* and increased *XBP-1, IRF-4,* and *J-chain*); however, the GFP$^+$Synd-1$^-$ cells appeared less differentiated, with residual *Bcl6*, *Pax5*, *AID*, and *CIITA* expression, as well as reduced IgM secretion (Fig. 7, E and G). This conclusion was supported by cell sorting experiments that indicated that, whereas GFP$^+$Synd-1$^+$ cells were fully differentiated and retained their phenotype after reculture, GFP$^+$Synd-1$^-$ cells were capable of self-renewal and differentiation into the GFP$^+$Synd-1$^+$ compartment (Fig. 7 F). Finally, a small number of transcripts including *Synd-1*, *bcl2*, *bcl6*, and most clearly *CXCR2* were differentially expressed between fractions 2 and 3, further highlighting the heterogeneity of the ASC phenotype (Fig. 7 G).

Discussion

Heterogeneity in the Plasma Cell Phenotype. The principal difficulties in analyzing ASCs are their rarity (<0.5% of lymphoid tissues) and that only the retrospective analysis of Ig secretion itself defines the cell type. The *Blimp*^{gfp} reporter allele described here provides us with a simple and extremely accurate methodology to identify all plasma cells in culture and, most importantly, from lymphoid organs in vivo.

A significant finding to come from the analysis of the *Blimp*^{gfp}-expressing ASCs is the heterogeneity of their generation, phenotype and function. There have been papers documenting heterogeneity in plasma cell phenotypes in mice (22) and humans (24, 25) although in the absence of a clear marker for ASCs, these studies are difficult to interpret. Several antigen combinations are commonly used as indicators of ASCs, including Synd-1, CD62L, CD43, CD38, and loss of B220 and CD19. Our analysis of ASCs from resting, immunized, and LPS-injected mice showed that, whereas all of these markers were altered, no combination identified all ASCs. For example, GFP$^+$ cells expressing a broad range of B220 and Synd-1 can be found in the BM and spleen. Moreover, the time course experiments after LPS injection showed clearly that recently induced ASCs have a distinct (B220$^+$Synd-1$^{int\text{-}high}$) phenotype that precedes the B220$^-$ Synd-1 positive or negative state.

The heterogeneity was also apparent in the level of *Blimp*^{gfp} expression, with approximately equal numbers of GFP^{int} and GFP^{hi} ASCs in the spleen and a predominance of GFP^{hi} cells in the BM (Fig. 2 B). We suggest that the GFP^{int} cells represent the more immature plasma cells that will undergo further differentiation to GFP^{hi} phenotype. This model was supported by the pronounced induction of GFP^{int} cells by LPS injection or immunization (Figs. 3 and 4). These cells had not completely lost CD19 or B220 expression, were short lived, and secreted antibody, whereas GFP^{hi} cells that had more completely down-regulated these markers were quiescent while also secreting Ig. We propose that these GFP^{int} cells represent the plasmablast stage of differentiation, a minority of which increase *Blimp-1* expression and enter the long-lived ASC compartment. It is also of note that, whereas blood ASCs are GFP^{int}, the BM ASCs are uniformly GFP^{hi}, suggesting that the increased *Blimp-1* expression associated with long-lived BM ASCs occurs after entry to the BM. Interestingly, the in vitro–generated ASCs continue to proliferate and have a similar fluorescence to the GFP^{int} stage in vivo, suggesting that these cells represent the plasmablast stage. Nothing is known about the regulation of *Blimp-1* expression levels in ASCs, but it is an intriguing prospect that stromal cell or antigen affinity determinants regulate entry into the long-lived plasma cell state via increasing *Blimp-1* expression.

The ability to differentiate B cells in vitro has enabled us to examine the extrinsic regulation of ASC heterogeneity. Perhaps most striking is the appearance of distinct Blimp^{gfp}-expressing populations after LPS stimulation that can be discerned based on the expression of Synd-1. GFP$^+$Synd-1$^-$ cells occur at a similar frequency to GFP$^+$Synd-1$^+$ ASCs but produce significantly less total Ig. The transcriptional profiles of the two populations were similar, with GFP$^+$Synd-1$^+$ cells displaying a trend toward greater divergence from the GFP$^-$ cells, including the down-regulation of *Pax5* and *Bcl6*, whereas the majority of chemokine receptors were co-coordinately regulated; CXCR2 was specifically expressed in the GFP$^+$Synd-1$^+$ cells. CXCR2 is the receptor for IL-8 and is not known to play a role in ASC biology. We have performed chemotaxis assays with recombinant IL-8, but, to date, have not been able to show any specific migration to-

ward this stimulus by GFP$^+$Synd-1$^+$ ASCs (unpublished data). A recent microarray paper has concluded that Blimp-1 activates distinct genetic programs in two B cell lines depending on the mode of stimulation (20). A more extensive analysis of the transcriptional profiles of the populations identified here will further test this possibility.

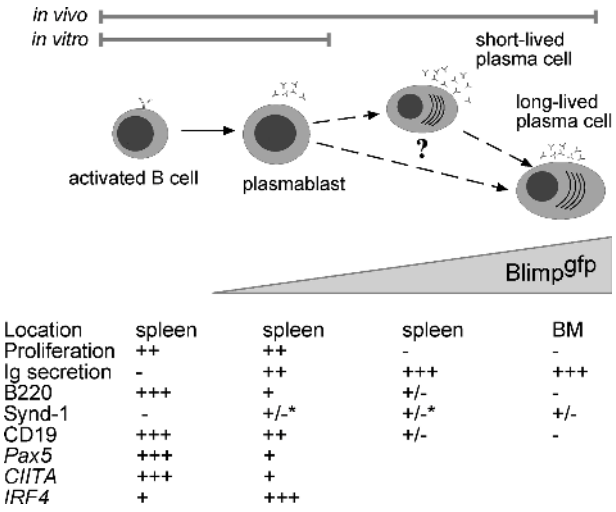
ASC heterogeneity also depended on the type of stimuli received, as the equal numbers of GFP$^+$Synd-1$^+$ and GFP$^+$Synd-1$^-$ cells produced in LPS cultures contrasted with the preponderance of GFP$^+$Synd-1$^+$ ASCs after CD40L and cytokine-driven differentiation. Previous in vivo studies have suggested that Synd-1$^-$ ASCs exist and are derived from Synd-1$^+$ precursors (22). Our results showed that this was not the case in vitro, as sorted GFP$^+$Synd-1$^+$ cells maintained their expression profile for several days after sorting, whereas GFP$^+$Synd-1$^-$ cells were capable of self-renewal as well as differentiation into Synd-1$^+$ cells. We suggest that there are two distinct pathways to GFP$^+$Synd-1$^+$ cells, one that proceeds through the GFP$^+$ Synd-1$^-$ intermediate and a second that is derived directly from the GFP$^-$ population. The existence of these two pathways was supported by the coincidence of their appearance during the time course of LPS induction (unpublished data). Whether Synd-1$^+$ cells give rise to negative cells in vivo is still to be determined, but the LPS injection experiments are compatible with this scenario.

Molecular Role of Blimp-1. A hallmark of plasma cell differentiation is the silencing of many of the genes associated with the mature B cell phenotype, whereas *Blimp-1* is activated by stimuli that induce an ASC fate (4). Blimp-1 functions primarily as a transcriptional repressor (11, 16, 21) that can recruit key players in gene silencing (12–14). However, cDNA microarray analysis also identified a cohort of transcripts that are induced by *Blimp-1* expression (20, 21). Direct promoter analyses have suggested that Blimp-1 represses *Pax5* (17), *c-myc* (16), and *CIITA* (36). This paper is the first to use the combination of a reporter of *Blimp-1* expression and Blimp-1–deficient cells to genetically determine the requirement for Blimp-1 in the regulation of a particular transcript.

A model of B cell terminal differentiation holds that mature B cells express the B cell maintenance factor *Pax5* and the oncogene *Bcl6*. Extrinsic signals such as cytokines or antigen result in the degradation of Bcl6, a repressor of *Blimp-1*. Blimp-1 activates differentiation to ASCs (30). Interestingly, *Pax5* was expressed in all GFP$^-$ populations, but was rapidly silenced in GFP$^+$ cells, supporting the notion that *Pax5* is repressed by Blimp-1 (17) and suggesting that this repression may be the pivotal transcriptional event in ASC differentiation. Indeed, as predicted by this model, the Pax5-repressed genes *XBP-1* (18) and *J-chain* (31) were silent and the Pax5-activated genes *AID* (32) and *CIITA* (33) were maintained in Blimp-1–deficient cells (Fig. 6 C).

Using several experimental systems, we and others have demonstrated that very low levels of Ig were produced by *Blimp-1*–deficient plasma cells (10). As Blimp-1 is not implicated directly in Ig transcription, it is likely that its role in inducing Ig production occurs via secondary proteins such as Pax5, IRF4, and XBP-1. Pax5 regulates Ig production by repressing *IgH* and *Igκ* expression (for review see reference 37), whereas IRF4 binds in the *Igκ 3′* and λ_{2-4} enhancers and is essential for Ig production (38–40). As Blimp-1–deficient B cells maintain Pax5 and lack IRF4, the inability to activate appropriate Ig transcription levels would limit the secretion rate and explain the inability of XBP-1 alone to rescue Ig secretion by *Blimp-1*$^{-/-}$ B cells as XBP-1 functions after high level Ig production has been initiated (10, 41). Together, these data suggest that the inability of *Blimp*$^{gfp/gfp}$ cells to repress *Pax5* and activate *IRF4* is sufficient to explain the decreased Ig expression associated with the phenotype.

Blimp-1 Expression Levels Control the Plasma Cell Terminal Differentiation Pathway. The *Blimp*^{gfp} allele described here allows the identification and characterization of all ASCs. Our data suggest that *Blimp-1* expression is induced in distinct phases; an intermediate expression level associated with short-lived plasmablasts and a more differentiated, long-lived, Blimp-1–high phenotype. Although a broad heterogeneity of the plasma cell lineage is apparent at the level of cell surface phenotype, Ig secretion, and transcriptional profiles, these are significantly correlated with expression levels of *Blimp-1*. These data suggest a model of cellular ontogeny where increasing Blimp-1 levels result in progressive maturation of ASCs (Fig. 8). Short-lived plasmablasts in the spleen in vivo and in vitro, characterized by low Blimp-1 levels, which have not completely extinguished the mature B cell expression profile, proliferate and secrete Ig. In contrast, long-lived, noncycling plasma cells in the spleen and BM are associated with higher Blimp-1 levels and have more completely down-regulated expres-



Location	spleen	spleen	spleen	BM
Proliferation	++	++	-	-
Ig secretion	-	++	+++	+++
B220	+++	+	+/-	-
Synd-1	-	+/-*	+/-*	+/-
CD19	+++	++	+/-	-
Pax5	+++	+		
CIITA	+++	+		
IRF4	+	+++		

Figure 8. Schematic of plasma cell terminal differentiation based on increasing Blimp-1 expression. Relative function or expression status of several parameters is indicated on an arbitrary scale. Dotted lines indicate hypothetical pathways. Analysis of *Pax5, IRF4,* and *CIITA* expression is based in vitro evidence only. *, Synd-1 expression on plasma cells is heterogeneous and context specific (see Discussion).

sion of B lineage proteins and genes, but retain some heterogeneity for known plasma cell markers. Although it is not yet possible to ascertain if a linear relationship between Blimp-1 levels and cellular differentiation exists, the Blimp^{gfp} model provides us with the a valuable tool to resolve this question.

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