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 an 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 of a functional Blimp-1 allele.

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(hydroxy-3)-nitrophenyl acetyl; Synd-1, syndecan-1.
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<sup>gfp</sup> 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<sup>gfp</sup> defines the ontogeny of B cells from plasmablasts to long-lived plasma cells.

Materials and Methods

**Generation of the Blimp<sup>gfp</sup> 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), gEFp cDNA, and a SV40 polyadenylation signal. The PGK-Neo<sup>r</sup> 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–I–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 Spel (3′ arm) and hybridized to a 500-bp fragment of PCR-amplified genomic DNA 5′ to the homology arms (see Fig. 1 A). C57BL/6 DNA gave a band of 3.8 kb, whereas correctly targeted clones gave an additional 4.5-kb band. PCR genotyping was performed using the primer combination: bl-1 5′-GGCAAGATCGAATGAGTGC-3′, bl-2 5′-TGAGTGTCGACAATGACCCA-3′, and bl-3 5′-GCCGAATTCTTATATGACCCA-3′. PCR fragments of 611 and 531 bp were indicative of wild-type and targeted alleles, respectively.

**Transplantation of Fetal Liver Cells.** Blimp<sup>gfp/+</sup> (C57BL/6 Ly5.2) mice were intercrossed; fetal liver cells were isolated from E14.5 embryos and genotyped by PCR, and 1–3 × 10<sup>7</sup> fetal liver cells were injected into lethally irradiated C57BL/6 Rag<sup>−/−</sup> Ly5.1 congenic recipients (2 × 350 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), MHCI (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 LSRII 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<sup>+</sup> 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 streptavidininhorseradish 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<sup>gfp/+</sup> mice, and animals were analyzed daily for up to 7 d. Immunization was with a single i.p. injection of 100 μg (4-hydropyran-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<sup>+</sup> populations was determined using NP-specific ELISPOT (26).

**In Vitro Cell Culture.** Naive B cells were purified from spleens by T cell complement depletions, Percoll gradient centrifugation, and B220 magnetic bead purification (Miltenyi Biotec) as described previously (29). Purified cells (95% IgM<sup>+</sup> IgD<sup>−</sup>B220<sup>+</sup>) were cultured at 10<sup>5</sup> cells/ml with optimal concentrations of CD40L, 500 U/ml IL-4, and 2 ng/ml IL-5. 4 × 10<sup>5</sup> cells/ml were used for 20 μg/ml LPS stimulation. Cell membranes expressing mouse CD40L were prepared from S21 cells (42). Recombinant 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-<sup>3</sup>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<sup>gfp</sup> 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<sup>gfp</sup> allele.
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 $\sim 10^5$-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$^{miu}$) 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$^{miu}$ 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
the majority of Synd-1− cells being GFPint (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-1 expression occurred in GFPintB220+ cells and GFP+ ASC from spleen (LPS induced and resting) and BM. GFP+ cells left of the dividing line were considered GFP intermediate (GFPint) and those to the right were considered GFP high (GFPhi). (C) Analysis of the surface phenotype of gated cells from B. Mean fluorescence index is indicated for each histogram. (D) Sorted GFPint and GFPhi cells were sorted as for B and subjected to semi-quantitative RT-PCR analysis. Serial fivefold dilutions of the cDNA were analyzed. (E) BM GFPint Synd-1− and GFPhi Synd-1+ cells were sorted and assayed for Synd-1 mRNA. HPRT was a loading control. 

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
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 GFPint and GFPhi, it was apparent that there was a rapid increase in the GFPint population in the context of a relatively stable GFPhi population (Fig. 4 C). Comparison of these populations revealed a high frequency of NP-specific IgG1 ASCs...
in the GFP\textsuperscript{int} population, compared with the GFP\textsuperscript{hi} population, although the frequency in the GFP\textsuperscript{hi} compartment increased over time (Fig. 4 D). These data suggest that the GFP\textsuperscript{int} population is the rapidly expanding plasmablast population localized in the splenic foci, whereas the GFP\textsuperscript{hi} ASCs appear to be the more stable, long-lived ASC compartment of the spleen. The frequency of Synd-1\textsuperscript{+}GFP\textsuperscript{+} 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\textsuperscript{hi}, we could only detect GFP\textsuperscript{int} cells in the blood (Fig. 4 A). These results agreed with the appearance of GFP\textsuperscript{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\textsuperscript{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\textsuperscript{int} cells had turned over, whereas very few GFP\textsuperscript{hi} cells in either spleen or BM were cycling (Fig. 5). It is interesting to note that the frequency of GFP\textsuperscript{+} cells in BM did not change as a result of immunization, even though the frequency of cycling splenic GFP\textsuperscript{int} and GFP\textsuperscript{hi} cells increased markedly (Fig. 4, A and B, and not depicted). In summary, the induction of GFP\textsuperscript{int} population by both polyclonal and antigen-specific immunization, in the context of a relatively stable population of long-lived GFP\textsuperscript{hi} cells, suggests a plasma cell maturation pathway reflected by levels of Blimp-1 expression and that the GFP\textsuperscript{int} cells are plasmablasts.

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

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**Figure 5.** GFP\textsuperscript{int} cells are short-lived plasmablasts. Blimp\textsuperscript{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.

**Figure 6.** Induction of ASC differentiation in vitro requires Blimp-1. (A) Wild-type, Blimp\textsuperscript{gfp/+}, and Blimp\textsuperscript{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\textsuperscript{+} 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\textsuperscript{+}; (–) GFP\textsuperscript{−}. The Blimp-1 proteins and the membrane-bound (\(\mu M\)) and secreted IgM (\(\mu S\)) 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\textsuperscript{gfp/gfp} cells do not initiate the ASC transcriptional cascade and remain indistinguishable from the GFP\textsuperscript{−} 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**+/+ cultures contained a population of GFP+ cells that were absent in the wild-type cultures (Fig. 6 A). Notably, **Blimp**−/− 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**−/− B cells, although normally capable of proliferating and class switching in response to exogenous stimuli, secreted little antibody compared with **Blimp**+/+ or wild-type cultures (unpublished data). Western blotting of sorted GFP+ cells from **Blimp**+/+ cultures confirmed that Blimp-1 expression correlated with high levels of IgH (μS) as compared with GFP− cells from the same cultures. In contrast, although BlimpΔ 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** 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**+/+ cells and the lack of **Blimp-1** exons 7–8 transcripts in **Blimp**−/− 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**−/− 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** identifies a heterogeneous population of ASC in vitro.](image-url)
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<sup>gfp</sup> B cells revealed this was indeed the case, as only ~50% of the GFP<sup>+</sup> cells were Synd<sup>−</sup>-1<sup>−</sup>. In contrast, most (>85%) GFP<sup>+</sup> cells were Synd<sup>−</sup>-1<sup>−</sup> in CD4<sup>+</sup>/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<sup>−</sup>Synd<sup>−</sup>-1<sup>−</sup> (fraction 1), GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>−</sup> (2), GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>+</sup> (3), and GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>−</sup> (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<sup>+</sup> fractions (Fig. 7 D). Interestingly, the rate of IgM secretion was reproducibly lower in the Synd<sup>−</sup>-1<sup>−</sup> fraction2 compared with those cells expressing Synd<sup>−</sup>-1 (Fig. 7 E). It has been reported that Blimp<sup>−</sup>-1<sup>−</sup> 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<sup>+</sup> populations displayed the hallmarks of ASC differentiation (decreased Pax5, AID, CIITA and increased XBP-1, IRF-4, and J-chain); however, the GFP<sup>−</sup>Synd<sup>−</sup>-1<sup>−</sup> 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<sup>+</sup>Synd<sup>−</sup>-1<sup>+</sup> cells were fully differentiated and retained their phenotype after reculture, GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>−</sup> cells were capable of self-renewal and differentiation into the GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>−</sup> compartment (Fig. 7 F). Finally, a small number of transcripts including Synd<sup>−</sup>-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<sup>gfp</sup> 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<sup>gfp</sup>-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<sup>+</sup> 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<sup>+</sup>Synd<sup>−</sup>-1<sup>−</sup><sub>int-high</sub>) phenotype that precedes the B220<sup>+</sup>Synd<sup>−</sup>-1 positive or negative state.

The heterogeneity was also apparent in the level of Blimp<sup>gfp</sup> expression, with approximately equal numbers of GFP<sup>+</sup> and GFP<sup>+</sup> ASCs in the spleen and a predominance of GFP<sup>+</sup> cells in the BM (Fig. 2 B). We suggest that the GFP<sup>+</sup> cells represent the more immature plasma cells that will undergo further differentiation to GFP<sup>+</sup> phenotype. This model was supported by the pronounced induction of GFP<sup>+</sup> 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<sup>+</sup> cells that had more completely down-regulated these markers were quiescent while also secreting Ig. We propose that these GFP<sup>+</sup> cells represent the plasmablást 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<sup>+</sup>, the BM ASCs are uniformly GFP<sup>+</sup>, 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<sup>+</sup> stage in vivo, suggesting that these cells represent the plasmablást 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<sup>gfp</sup>-expressing populations after LPS stimulation that can be discerned based on the expression of Synd-1. GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>−</sup> cells occur at a similar frequency to GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>+</sup> ASCs but produce significantly less total Ig. The transcriptional profiles of the two populations were similar, with GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>−</sup> cells displaying a trend toward greater divergence from the GFP<sup>+</sup> cells, including the down-regulation of Pax5 and Bcl6, whereas the majority of chemokine receptors were coordinately regulated; CXCR2 was specifically expressed in the GFP<sup>+</sup>Synd<sup>−</sup>-1<sup>−</sup> 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 the 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− 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. Extrinsinc 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 Igk expression (for review see reference 37), whereas IRF4 binds in the Igκ 3′ and λ5 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−/− 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−/− 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-

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**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).
Heterogeneity in Plasma Cell Differentiation

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