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Initiation of Plasma-Cell Differentiation Is Independent of the Transcription Factor Blimp-1

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SUMMARY

Blimp-1 is considered an essential regulator of the terminal differentiation of B cells into antibody-secreting plasma cells. We show here that *Rag1*^{-/-} mice reconstituted with fetal liver cells homozygous for a DNA-binding-deficient mutant of *Prdm1* (the gene encoding Blimp-1) lack a defined plasma-cell compartment, yet show detectable amounts of all immunoglobulin isotypes. In vitro analysis revealed that Blimp-1 is not required for the initiation of antibody secretion but is essential for subsequent high immunoglobulin production. Blimp-1-independent differentiation was blocked at a preplasmablast stage characterized by decreased *Pax5* expression and the activation of plasma-cell genes. Analysis of Blimp-1-sufficient differentiation revealed a phase prior to Blimp-1 expression in which several genes normally repressed by *Pax5* are re-expressed, suggesting that plasma-cell differentiation is initiated by the inhibition of *Pax5* function. Our results indicate that full plasma-cell differentiation but not commitment to the plasma-cell fate requires the expression of functional Blimp-1.

INTRODUCTION

The differentiation of B cells into antibody-secreting cells (ASC) is a crucial component of the immune response. After encountering antigen, B cells undergo multiple rounds of division and have the ability to differentiate into plasmablasts, plasma cells, or memory B cells (Manz et al., 2005). Plasmablasts are short-lived cycling ASC that are found in extrafollicular foci in peripheral lymphoid organs and resemble ASC generated after in vitro stimulation. In contrast, long-lived plasma cells are noncycling, found preferentially in the bone marrow, and derive predominantly from germinal centers (GC) (Manz et al., 2005). The GC reaction also produces memory B cells that can rapidly differentiate into ASC after re-exposure to antigen.

A small number of transcription factors have been identified that guide the developmental program leading to ASC differentiation (Kallies and Nutt, 2007; Shapiro-Shelef and Calame, 2005). Interestingly, the evidence to date suggests that this gene-regulatory network is dominated by transcriptional repression. One group of factors, including *Pax5*, *Mitf*, *Bach2*, and *BCL6*, are expressed in activated B cells and act predominantly by repressing differentiation (reviewed in Shapiro-Shelef and Calame, 2005). *Pax5* represses genes associated with the stem cell and non-B lineage programs as well as a number of genes involved in ASC differentiation, including *Prdm1* (the gene encoding B lymphocyte-induced maturation protein-1 [Blimp-1]), *Xbp1*, and *Igj* (*J chain*) (Delogu et al., 2006; Nera et al., 2006; Reimold et al., 1996; Rinkenberger et al., 1996). *BCL6* and *Bach2* suppress ASC development in part by repressing *Prdm1* (Ochiai et al., 2006; Tunyaplin et al., 2004), whereas *Mitf* represses another key regulator of the ASC lineage, *Irf4* (Lin et al., 2004).

The molecular mechanism by which this repression cascade is overcome to allow ASC formation remains unclear, but it is known that differentiation and high immunoglobulin (Ig) secretion require IRF-4, XBP-1, and Blimp-1 (Shapiro-Shelef and Calame, 2005). IRF-4 is present at low amounts throughout the B cell lineage, where it regulates isotype switching. Upon differentiation, IRF-4 expression markedly increases, which is essential for ASC formation and Ig secretion (Klein et al., 2006; Sciammas et al., 2006). XBP-1 is an ubiquitously expressed transcription factor that directly controls aspects of the secretory pathway and is strongly induced in ASC by a combination of loss of *Pax5*-mediated gene repression and posttranscriptional control by the unfolded protein response (UPR) in the endoplasmic reticulum (Iwakoshi et al., 2003; Reimold et al., 1996). Finally, ASC development is also critically dependent on Blimp-1, which has been termed the “master regulator” of plasma-cell differentiation.

Blimp-1 is expressed in ASC from human and mouse but is absent from earlier stages of B cell ontogeny (Angelini-Duclos et al., 2000; Kallies et al., 2004). Many studies have contributed to the view that Blimp-1 is essential for Ig secretion and ASC development. Notably, ectopic Blimp-1 is sufficient to induce differentiation (Turner et al., 1994), whereas the B cell-specific deletion of *Prdm1* in mice demonstrates a requirement for Blimp-1 in the generation

of a functional ASC compartment (Savitsky and Calame, 2006; Shapiro-Shelef et al., 2003). Blimp-1-deficient mice show reduced, but not absent, serum Ig and display severely diminished antibody responses after immunization, whereas inactivation of *Prdm1* in preformed plasma cells leads to the loss of this lineage (Shapiro-Shelef et al., 2003, 2005). Gene-expression studies have shown that Blimp-1 is essential to extinguish many aspects of the mature B cell gene-expression program, including silencing of *Myc* (the gene encoding *c-myc*) and for the exit from the cell cycle characteristic of terminal differentiation (Shaffer et al., 2002). Blimp-1 also directly binds and represses the promoters of *Pax5*, and potentially of *Bcl6*, thereby providing a mechanism by which its induction leads to ASC differentiation and loss of the B cell phenotype (Lin et al., 2002; Vasanwala et al., 2002).

To investigate the function of Blimp-1 in ASC, we introduced *gfp* into the *Prdm1* locus (Kallies et al., 2004). In heterozygous mice, quantitative changes of Blimp-1 expression, revealed by GFP, can be used to define distinct stages in ASC ontogeny, with intermediate expression associated with short-lived plasmablasts and high expression with long-lived plasma cells (Blink et al., 2005; Kallies et al., 2004). We have now used this mouse strain to investigate the earliest stages of ASC formation and show here that Blimp-1 is dispensable for the initiation of the ASC differentiation program. We propose that downmodulation of *Pax5* activity is the critical step that leads to the formation of a preplasmablast that is capable of secreting low amounts of Ig independent of Blimp-1 function. Blimp-1 is, however, essential for the progression from this earliest stage in ASC formation leading to the full manifestation of the transcriptional program and high-titer antibody production.

RESULTS

Low Ig Secretion Occurs In Vivo in the Absence of Blimp-1

By using *Prdm1^{+gfp}* mice, we have shown that Blimp-1 is expressed in all ASC (Kallies et al., 2004) and, at a lower level, in antigen-experienced T cells (Kallies et al., 2006). Mice homozygous for the mutant allele die in utero, so we generated chimeric mice by reconstituting irradiated *Rag1^{-/-}* mice with *Prdm1^{gfp/gfp}* fetal liver cells as described (Kallies et al., 2004), called *Prdm1^{gfp/gfp}* mice hereafter. The *Prdm1^{gfp}* allele expresses GFP and a truncated Blimp-1 protein that is deficient in DNA binding and non-functional, and therefore, this strategy enabled us to follow B lymphopoiesis in the absence of wild-type Blimp-1.

ASC could be detected readily in the spleen of *Prdm1^{+gfp}* mice by their intermediate to high GFP expression (Kallies et al., 2004). The majority of these cells expressed the ASC marker Syndecan-1 (Synd-1 or CD138) and were low for CD19 (Figure 1A). In contrast, *Prdm1^{gfp/gfp}* mice lacked Synd-1 expression on GFP⁺ cells suggesting an absence of ASC (Figure 1A). In these mice, however, we detected an increased population of GFP⁺Synd-1⁻ cells. Although a large fraction of these

cells were T cells (Kallies et al., 2006), up to 50% were CD19⁺ and belonged to the B cell lineage (Figure 1A). ELISA analysis of serum showed that all Ig classes were detectable in *Prdm1^{gfp/gfp}* mice but were reduced to between 0.1%–10% of controls (Figure 1B). Immunoprecipitation of IgG from serum of these mice confirmed the low amounts of Ig determined by ELISA (Figure 1C). This Ig arose in *Rag1^{-/-}* mice reconstituted with hematopoietic cells that were uniformly deficient for the Blimp-1 DNA-binding domain, so these data demonstrated that some Ig secretion occurred in the absence of wild-type Blimp-1.

The presence of Ig in the serum of *Prdm1^{gfp/gfp}* mice raised the question of whether this Ig derived from a distinct ASC population that was less dependent on Blimp-1 for its function. One isotype that appeared less affected by the loss of Blimp-1 was IgA (Figure 1B). IgA is produced mainly by specialized mucosal ASC of the gastro-intestinal tract, so we tested whether these cells were functional in the absence of Blimp-1 by assaying fecal IgA by ELISA and mucosal IgA by immunohistochemistry. Fecal IgA amounts in *Prdm1^{gfp/gfp}* mice were reduced to 1% of *Prdm1^{+gfp}* controls, but well above amounts found in *Rag1^{-/-}* mice (Figure 1D). We were, however, unable to locate any IgA-secreting cells in the lamina propria despite the presence of IgA-switched B cells in intestinal follicles (Figure 1E). These experiments demonstrated through a number of approaches that *Prdm1^{gfp/gfp}* mice lacked bona fide ASC but contain cells that were capable of low-titer Ig secretion.

Severely Impaired ASC Differentiation in Immunized Blimp-1 Mutant Mice

We have shown previously that T cell-dependent (NP-KLH) and -independent (LPS) antigens induce a transient plasmablast population with lower Blimp-1 expression than that of mature plasma cells (Kallies et al., 2004). In order to examine whether this state could be induced in the absence of Blimp-1, we injected *Prdm1^{gfp/gfp}* or control *Prdm1^{+gfp}* mice with LPS and analyzed the spleens at days 3 and 7. As expected, in *Prdm1^{+gfp}* mice, a substantial population of GFP^{int}Synd-1⁺ plasmablasts was induced by day 3. This population had resolved almost completely by day 7, but substantially increased serum IgM amounts were detectable by ELISA at this time point. In contrast, no GFP⁺ plasmablasts or increased serum IgM titers were detected in *Prdm1^{gfp/gfp}* mice, suggesting that the *Prdm1^{gfp/gfp}* B cells failed to differentiate in response to T-independent stimuli (Figure S1A in the Supplemental Data available online).

In a second approach, we examined the development of ASC in response to immunization with the T-dependent antigen NP-KLH. In this model, NP-specific ASC can be found initially in the spleen and appear at later time points in the GFP^{high} plasma-cell population in the spleen and bone marrow (Blink et al., 2005; Kallies et al., 2004). Immunization of *Prdm1^{gfp/gfp}* mice led to the formation of GCs in spleen and the generation of antigen-specific memory B cells (defined as NP binding, IgG1⁺B220⁺CD38^{high}Synd-1⁻), which were comparable in numbers and frequency

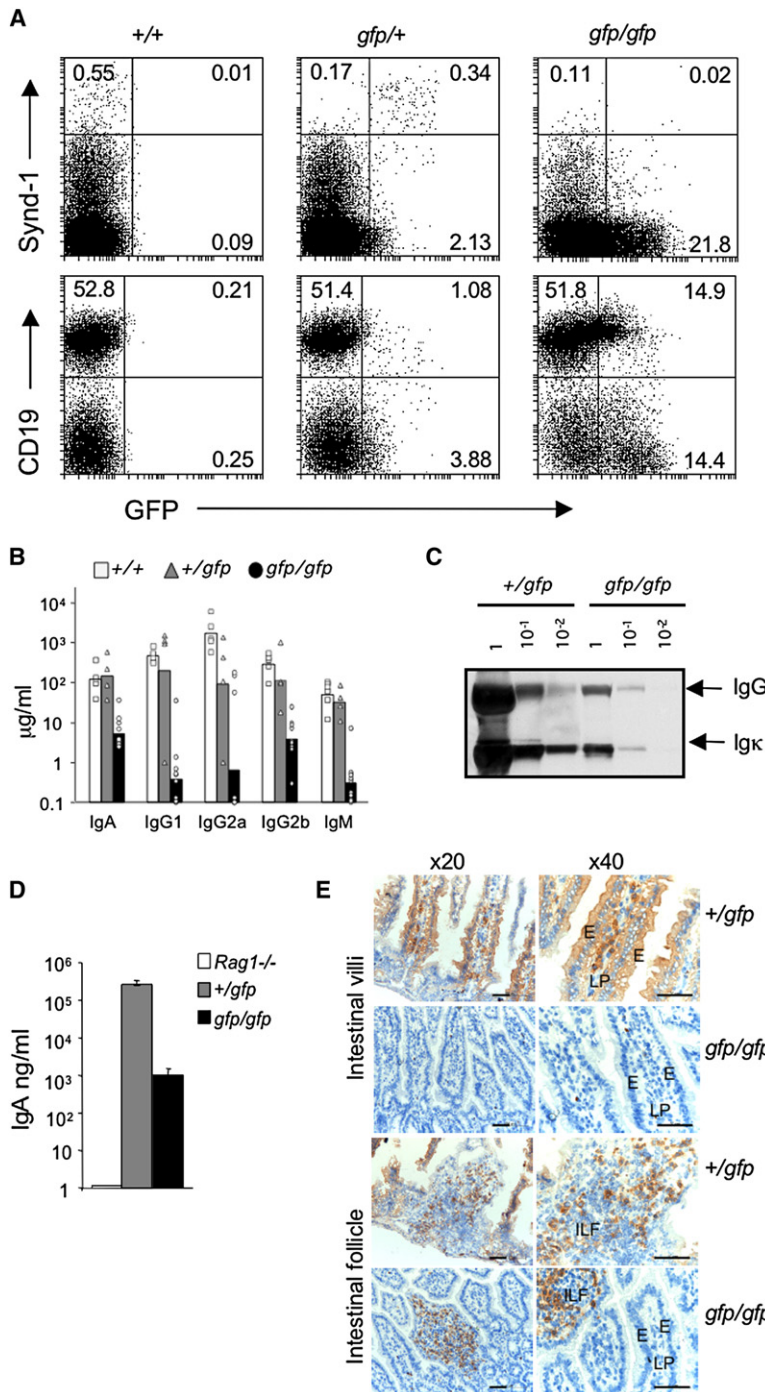


Figure 1. Blimp-1 Is Required for Most but Not All Antibody Secretion In Vivo

(A) Flow cytometric analysis of spleens from *Rag1*^{-/-} mice reconstituted with E14.5 fetal liver cells of the indicated genotypes. Numbers indicate the percentage of cells in each quadrant. Data are representative of at least three experiments.

(B) Serum Ig titers of individual mice as in (A) were determined by ELISA. Bar graphs represent the means.

(C) Immunoprecipitation and immunoblotting of serum IgG and Igκ from *Prdm1*^{+/*gfp*} and *Prdm1*^{*gfp*/*gfp*} mice in serial dilution as indicated. A representative of two experiments is shown.

(D) Fecal IgA amounts of mice as in (A) were assayed by ELISA. Data are mean derived from five mice of each genotype ± SD.

(E) Immunohistochemical staining for mucosal IgA. Although both genotypes showed normal switching to IgA, staining brown, within the isolated lymphoid follicles (ILF), *Prdm1*^{*gfp*/*gfp*} mice lacked IgA⁺ ASC in the lamina propria (LP) and IgA staining in the epithelial cells (E) responsible for the transport of IgA to the gut lumen. Scale bars represent 50 µm. Data are representative of four mice of each genotype.

of somatic hypermutations between *Prdm1*^{*gfp*/*gfp*} and wild-type mice (data not shown). In keeping with published data (Shapiro-Shelef et al., 2003), no isotype-switched, NP-specific ASC were found in the bone marrow of immunized *Prdm1*^{*gfp*/*gfp*} mice, although small ELISpots were detected in the spleen at each time point (Figure S1B). These data again provide evidence for the induction of low specific Ig secretion in the absence of Blimp-1.

ASC Differentiation Is Induced but Abortive in the Absence of Blimp-1 In Vitro

To further investigate the dependence of Ig production on Blimp-1, purified resting splenic B cells were cultured in conditions that mimic T cell help (anti-CD40+IL4+IL5) or microbial stimuli (LPS) for 4 days and then analyzed for ASC phenotype and Ig production (Hasbold et al., 2004; Kallies et al., 2004). As expected, the *Prdm1*^{+/*gfp*} cultures contained GFP⁺ cells (Figure 2A), which we have shown to

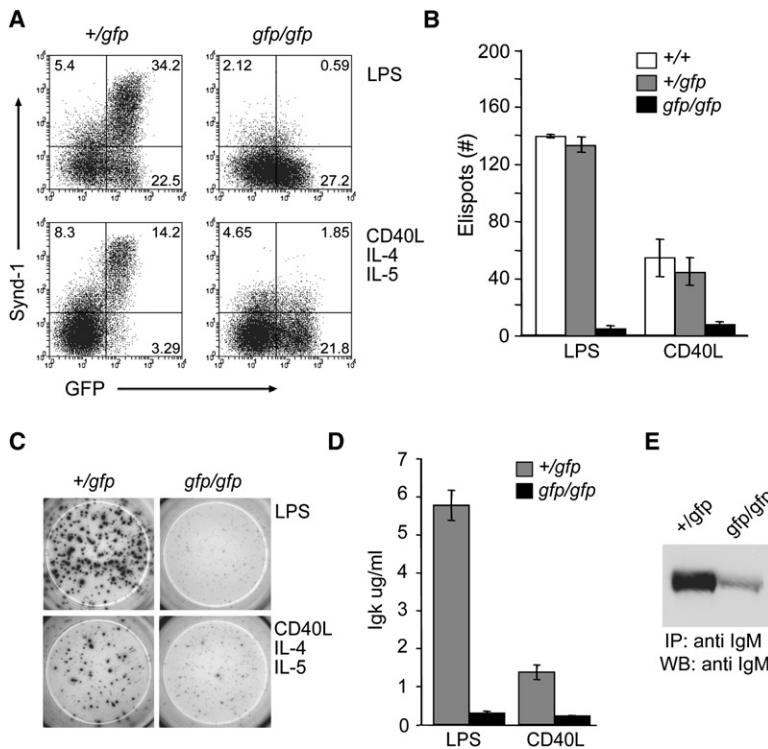


Figure 2. Antibody Secretion In Vitro in the Absence of Functional Blimp-1

(A) Resting splenic B cells of the indicated genotypes were cultured in the presence of LPS or a combination of anti-CD40+IL4+IL5 for 4 days and examined for GFP and Synd-1 expression.

(B) Cultured B cells as in (A) were assayed for Ig κ secretion by ELISpot. Data represent the mean numbers of ELISpots per 200 cells from triplicate wells \pm SEM.

(C) Representative ELISpot wells showing predominantly small spots in *Prdm1^{gfp/gfp}* cultures.

(D) Total Ig κ production from cultures as in (A). Data are presented as the mean of samples assayed in triplicate \pm SD. Data in (A)–(D) are representative of at least three independent experiments.

(E) Supernatants of unsorted *Prdm1^{+/gfp}* and *Prdm1^{gfp/gfp}* B cells cultured in LPS were immunoprecipitated and subjected to immunoblotting with an anti-IgM. A representative of two independent experiments is shown.

be ASC regardless of their Synd-1 expression status (Kallies et al., 2004). GFP was also detected in *Prdm1^{gfp/gfp}* B cell cultures, but these cells lacked Synd-1 (Figure 2A). ELISpot analysis confirmed the presence of large numbers of ASC in the *Prdm1^{+/gfp}* B cell cultures, but ELISpots were also produced from cultures of *Prdm1^{gfp/gfp}* B cells (Figure 2B). Whereas *Prdm1^{+/gfp}* or wild-type cultures yielded mainly large and prominent ELISpots, those generated by *Prdm1^{gfp/gfp}* B cell cultures were smaller and less pronounced (Figure 2C). The presence of low-titer Ig in the supernatants of *Prdm1^{gfp/gfp}* cultures was confirmed by ELISA (Figure 2D). Interestingly, B cells cultured in anti-CD40+IL4+IL5 were less affected by the loss of Blimp-1 function than those cultured in the presence of LPS. To exclude nonspecific mechanisms of Ig release, such as shedding of membrane-bound IgM into the supernatant, we immunoprecipitated IgM from LPS-stimulated B cell cultures. Immunoblotting showed no difference in the molecular weight of the IgM derived from either culture, indicating that Blimp-1 mutant cells produce low amounts of the secreted form of IgM (Figure 2E).

The expression of GFP in stimulated mutant B cells suggested that they had initiated ASC differentiation and that these cells were the source of the Ig observed in *Prdm1^{gfp/gfp}* cultures. To confirm this assumption, we directly compared populations sorted on the basis of GFP and Synd-1 expression from anti-CD40+IL4+IL5-stimulated cultures. Interestingly, a similar degree of B220 downregulation was observed in GFP⁺Synd-1⁻ cells from each genotype, suggesting that these cells had indeed initiated ASC differentiation (Figure 3A). Sorting of GFP⁺ cells from *Prdm1^{+/gfp}* and *Prdm1^{gfp/gfp}* cultures confirmed that

GFP expression correlated with Ig secretion (Figures 3B and 3D). Importantly, GFP⁺Synd-1⁻ cells from either culture yielded similar numbers of ELISpots (Figures 3B and 3C) and secreted similar amounts of total Ig on a per cell basis (Figure 3D). In line with earlier observations that B cells stimulated by LPS can differentiate into GFP⁺Synd-1⁺ via a GFP⁺Synd-1⁻ stage (Kallies et al., 2004), sort and reculture experiments showed that GFP⁺Synd-1⁻ *Prdm1^{+/gfp}* cells found in anti-CD40+IL4+IL5 cultures were precursors of fully differentiated GFP⁺Synd-1⁺ ASC (Figure 3E).

Initiation of the Plasma-Cell Gene-Expression Program in the Absence of Blimp-1

To examine whether B cells cultured in anti-CD40+IL4+IL5 were able to regulate genes associated with ASC differentiation in the absence of functional Blimp-1, we sorted GFP⁺ and GFP⁻ cells according to the gates depicted in Figure 3A and performed semiquantitative RT-PCR analysis. Surprisingly, we found that GFP⁺ *Prdm1^{gfp/gfp}* ASC had downregulated *Pax5* transcription and initiated expression of *Igj* and *Xbp1*, suggesting that early steps of ASC differentiation had occurred independently of Blimp-1 (Figure 3F). Similarly, the splicing of *IgH* mRNA into the secreted and membrane forms was regulated appropriately in both Blimp-1-sufficient and mutant GFP⁺Synd-1⁻ ASC. *Prdm1^{gfp/gfp}* B cells, however, failed to upregulate *Irf4* (Figure 3F).

The downregulation of *Pax5* was quantified by real-time RT-PCR, which showed a substantial decrease in *Pax5* in *Prdm1^{gfp/gfp}* GFP⁺Synd-1⁻ ASC, albeit to a lesser degree than in the equivalent population of *Prdm1^{+/gfp}* ASC (RNA

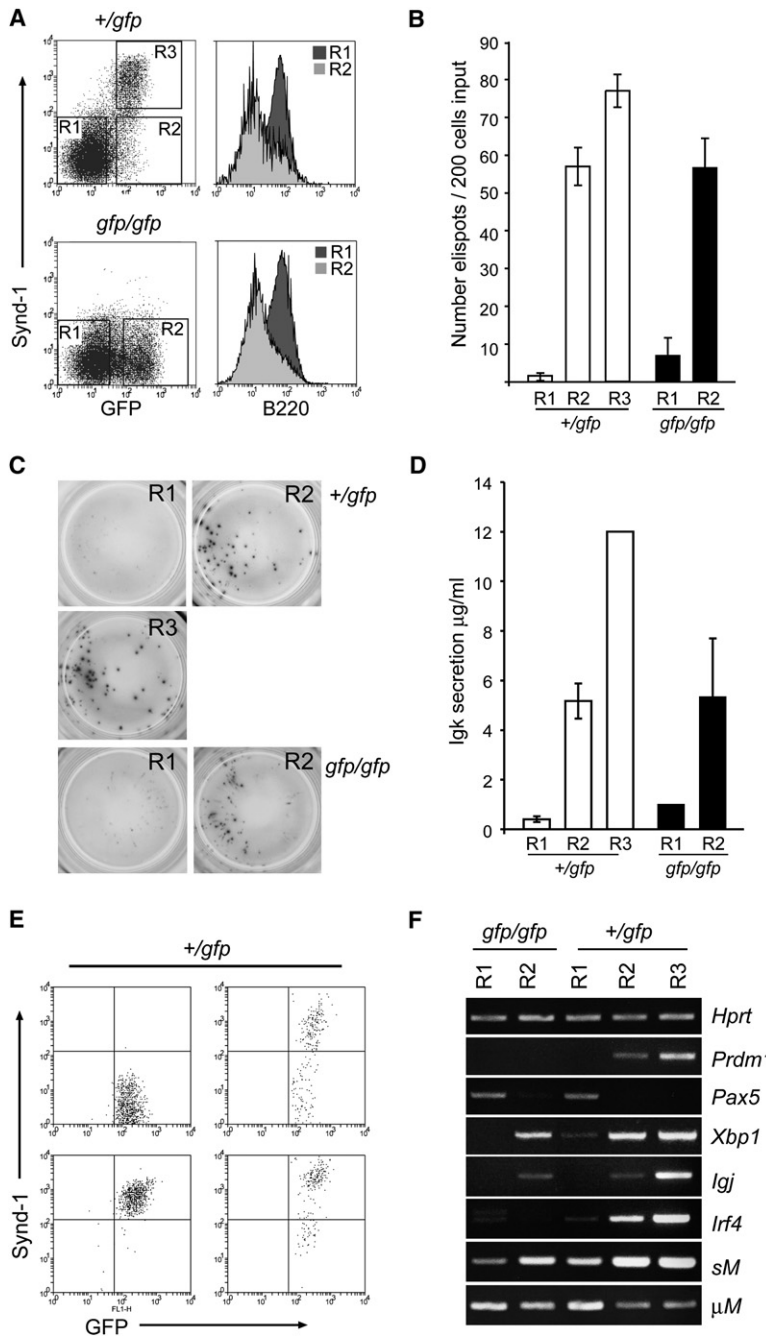


Figure 3. Initiation of ASC Differentiation in the Absence of Blimp-1 Function

Prdm1^{+/gfp} and *Prdm1*^{gfp/gfp} B cells were cultured in the presence of a combination anti-CD40+IL4+IL5 for 4 days and examined for GFP and Synd-1. Data in this figure are representative of 2–3 independent experiments.

(A) B220 expression was determined on cells gated as indicated.

(B–F) Cells of the indicated genotypes were sorted according to the gates depicted in (A). (B and C) Sorted populations were subjected to ELISpot assays with (B) showing the frequency of Igκ ELISpots (data represent the mean of triplicate wells ± SEM) and (C) showing representative ELISpot wells.

(D) Igκ secretion by equivalent numbers of the indicated populations as measured by ELISA. Data are the means ± SEM of triplicate measurements.

(E) R2 (GFP⁺Synd-1⁻) and R3 (GFP⁺Synd-1⁺) cells from *Prdm1*^{+/gfp} B cells cultured in anti-CD40+IL4+IL5 for 3 days were sorted and reanalyzed (left) or recultured for an additional 22 hr (right).

(F) Sorted populations were subjected to semi-quantitative RT-PCR analysis for the indicated genes. *Prdm1* primers span exons 7–8 (not expressed from the truncated allele).

expression reduced by 62.5% versus 84.1%, respectively, Figure 4A). In agreement with this, real-time RT-PCR confirmed the upregulation of the Pax5-repressed genes *Igj* and *Xbp1* in the Blimp-1 mutant ASC, although again this effect was less pronounced than in *Prdm1*^{+/gfp} ASC (Figure 4A). Importantly, the spliced form of *Xbp1* was also detected, suggesting the expression of functional XBP-1 in Blimp-1 mutant ASC (Figure 4C). Suppression of Pax5 protein expression was also evident, as was increased production of IgM and Igκ (Figure 4B). Thus, Blimp-1 mutant B cells, while not capable of differentiating

to fully secretory ASC, initiated the differentiation pathway that resulted in some Ig secretion in vitro.

IRF-4 has been shown to be essential for ASC differentiation, so we speculated that the lack of *Irf4* upregulation in *Prdm1*^{gfp/gfp} B cells was responsible for their failure to differentiate into high-titer Ig-secreting cells. However, retroviral overexpression of IRF-4 in *Prdm1*^{gfp/gfp} B cells was not sufficient to substantially increase Ig secretion, whereas, as expected, enforced expression of Blimp-1 resulted in markedly elevated Ig production (Figure 4D). Ectopic expression of IRF-4 also failed to induce *Igj*,

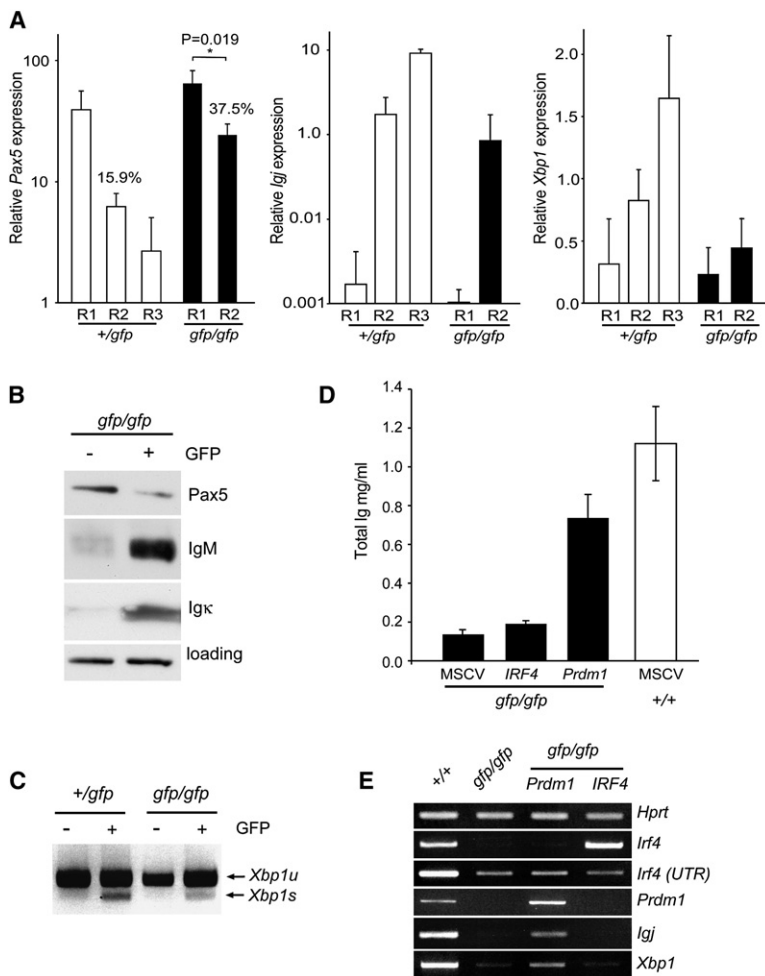


Figure 4. Initiation of the ASC Genetic Program in the Absence of Blimp-1

(A) Real-time PCR of populations sorted according to the gates depicted in Figure 3A. Data are presented as relative to *Hprt* mRNA. The mean \pm SEM of three biological replicates, each assayed in triplicate, is shown. Relative expression of *Pax5* mRNA in R2 compared to R1 are indicated (left). p values for statistical significance were derived by the paired Student's t test.

(B) Immunoblot analysis of populations as in (A). Protein extracts equivalent to 2×10^5 cells were loaded per lane. A nonspecific protein was used as a loading control. A representative of three experiments is shown.

(C) Cells of the indicated genotype were sorted according to GFP expression and subjected to RT-PCR. Primers detect the full-length unspliced (u) and spliced (s) forms of *Xbp1*. Data are representative of three independent biological replicates.

(D) Enforced expression of IRF-4 is not sufficient to rescue high Ig secretion in the absence of Blimp-1. Purified splenic B cells of the indicated genotypes were cultured in the presence of anti-CD40+IL4+IL5 and transduced with retrovirus encoding either *Irf4*, *Prdm1*, or the empty MCSV virus as control. After puromycin selection, live cells were cultured overnight and supernatants analyzed for total Ig by ELISA. Data are the mean \pm SEM of triplicate measurements and are representative of three independent experiments.

(E) Cells of the indicated genotypes either uninfected or transduced with *Prdm1*- or *Irf4*-expressing retroviruses were subjected to semi-quantitative RT-PCR analysis for the indicated genes. *Irf4* primers detect both endogenous and retrovirally derived transcripts whereas the *Irf4* (UTR) primers detect only the endogenous mRNA. A representative of three independent experiments is shown.

Xbp1, or endogenous *Irf4* in the absence of Blimp-1, whereas forced expression of Blimp-1 in *Prdm1^{gfp/gfp}* B cells resulted in increased amounts of *Igj* and *Xbp1* but was, surprisingly, not sufficient to induce *Irf4* (Figure 4E). These data suggest that Blimp-1 regulates factors other than *Irf4* to promote ASC differentiation.

Suppression of Pax5 Activity in B Cells Precedes Blimp-1 Expression

The observation that ASC differentiation was initiated in the absence of functional Blimp-1 was surprising and led us to examine the early events in this process upstream of Blimp-1. Pax5 is an essential regulator that maintains B cell identity and suppresses ASC differentiation (Delogu et al., 2006; Nera et al., 2006), so the downregulation of Pax5 represented an attractive candidate as the differentiation-initiating event. It has been proposed that Pax5 is a direct target of Blimp-1-mediated repression (Lin et al., 2002). The data described above, however, suggested that the initial downregulation of Pax5 was independent

of Blimp-1. To examine Pax5 activity in several different B cell populations, we made use of the recent finding that a number of target genes repressed by Pax5, including those encoding the receptor tyrosine kinase Flt3, the adhesion molecule embigin (Delogu et al., 2006; Holmes et al., 2006), and the inhibitor receptor PD-1 (Nutt et al., 1998), are re-expressed upon silencing of Pax5 during B cell differentiation. Freshly isolated resting splenic *Prdm1^{+gfp}* B cells expressed very low amounts of Flt3, embigin, and PD-1 (Figure 5A and data not shown). However, as early as 24 hr after culture in the presence of LPS or anti-CD40+IL4+IL5, expression of all three molecules was readily detected on a subset of stimulated B cells (data not shown). After 3 days, the majority of LPS-stimulated GFP⁻ B cells expressed Flt3, embigin, and PD-1, whereas a smaller fraction of anti-CD40+IL4+IL5-stimulated GFP⁻ B cells stained positive for these molecules (Figure 5A and data not shown). Importantly, the expression of these genes occurred in GFP⁻ cells, suggesting that derepression of Pax5 target genes preceded Blimp-1 induction.

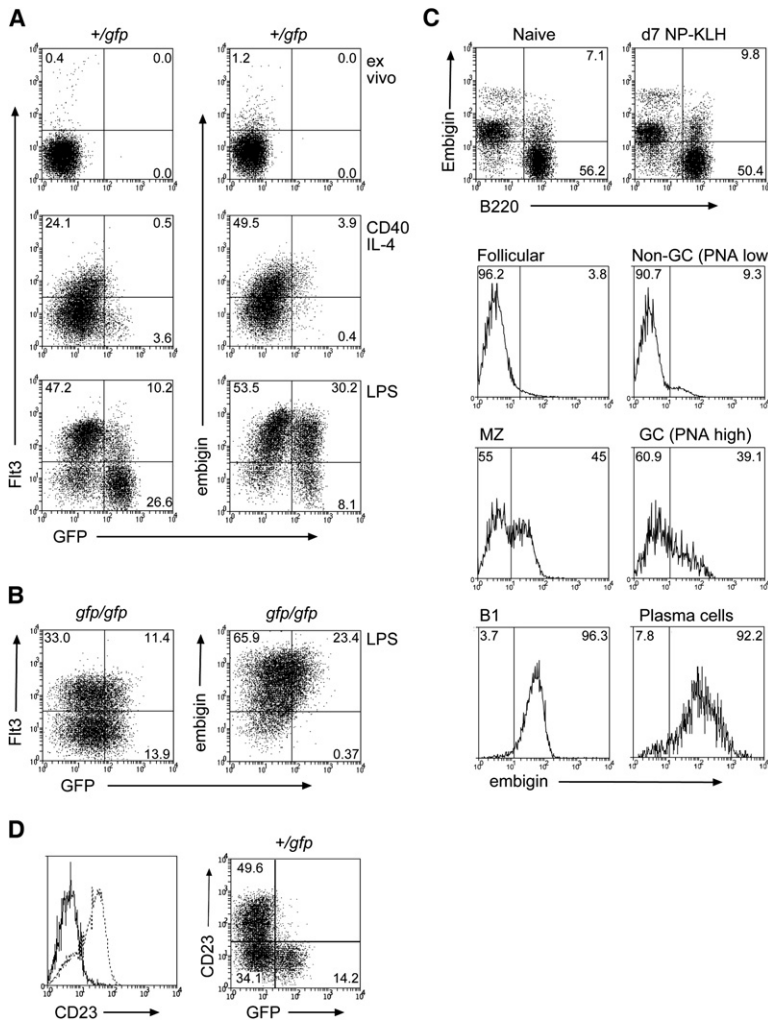


Figure 5. Blimp-1-Independent Changes in the Expression of Pax5 Target Genes in Activated B Cells

(A) Resting splenic B cells were isolated from *Prdm1^{+/gfp}* mice and cultured in the conditions indicated. Freshly isolated B cells and cells at day 3 of culture were analyzed for the expression of embigin or FIt3.

(B) Resting *Prdm1^{gfp/gfp}* splenic B cells were cultured in LPS for 3 days and stained for embigin and FIt3.

(C) Naive wild-type mice were analyzed for embigin expression in mature B cell subsets as indicated (left). Splenic B cells of mice immunized with NP-KLH were analyzed for embigin expression after 7 days (right). B cell subsets were defined as following: follicular B cells ($CD23^+CD21^{lo}IgM^{lo}$), MZ B cells ($CD23^-CD21^{hi}IgM^{hi}$), B1, peritoneal B1 B cells ($B220^{lo}CD5^+$). After immunization, B cells were defined as non-GC cells ($B220^+PNA^{low}$), GC cells ($B220^+PNA^{high}$). ASC from *Prdm1^{+/gfp}* mice were $Synd-1^{high}GFP^+$.

(D) Expression of CD23 on peritoneal B1 cells (left, solid line) in comparison to splenic ($B220^+$) B cells (left, broken line) and on splenic B cells from *Prdm1^{+/gfp}* mice cultured in LPS for 3 days (right). Results in this figure are representative of 2–4 independent experiments.

This interpretation was confirmed by the observation that activated *Prdm1^{gfp/gfp}* B cells also expressed these Pax5 target genes (Figure 5B).

To determine whether the release of Pax5-mediated gene repression occurred in vivo, we examined embigin expression in various mature B cell subsets in naive and immunized mice. Embigin staining was found constitutively on myeloid and splenic T cells whereas only a minority of splenic B cells was embigin⁺ (Figure 5C). A more detailed analysis of the splenic B cell compartment revealed that embigin was restricted to marginal zone (MZ) B cells, whereas follicular B cells were embigin⁻ (Figure 5C). In line with their predisposition to ASC differentiation (Fairfax et al., 2007), peritoneal B1 B cells were uniformly embigin⁺ (Figure 5C). The proportion of the embigin⁺ B cell population in spleen increased slightly after immunization with NP-KLH (Figure 5C), but the representation of embigin⁺ cells was markedly increased among the PNA^{high} GC cells and essentially all ASC were embigin⁺ (Figure 5C). Similar results were obtained with PD-1 and FIt3, although FIt3 was subsequently silenced in ASC by a distinct mechanism (Figure 5A and data not shown).

The re-expression of Pax5-suppressed genes prompted us to examine whether the expression of Pax5-activated genes, including *Cd23* (Visan et al., 2003), *Cd19*, *Cd79a* (Nutt et al., 1998), and *Blnk* (Schebesta et al., 2002) were also modulated in activated B cells. Indeed, we found that CD23 had a complementary expression profile to embigin, with low expression on peritoneal B1 and GC B cells when compared to splenic B cells, and that CD23 was markedly decreased when B cells were activated in vitro (Figure 5D). Molecular analysis further demonstrated that downregulation of *Cd79a* and *Blnk* occurred in $FIt3^+GFP^-$ B cells as well as peritoneal B1 cells (Figure 6A).

The modulation of Pax5 function in activated B cells could be induced by transcriptional or posttranslational regulation of Pax5. To distinguish between these possibilities, we sorted *Prdm1^{+/gfp}* B cells from LPS cultures according to GFP and FIt3 expression and subjected nuclear protein extracts to immunoblotting (Figure 6B). The amount of Pax5 was similar in all GFP^- subsets, irrespective of FIt3 expression, whereas Pax5 protein was downregulated in both GFP^+ fractions that, as expected, expressed Blimp-1, suggesting that the alleviation of the

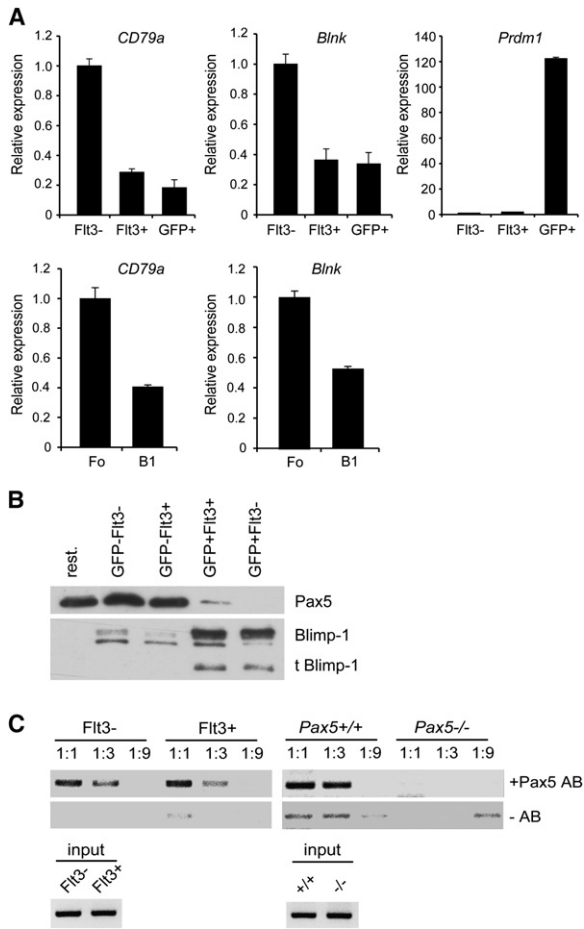


Figure 6. Diminished Expression of Pax5-Activated Genes in B Cell Subsets and Unchanged Pax5 DNA Binding in Cultured B Cells

(A) Real-time RT-PCR analysis of LPS-cultured splenic B cells sorted from *Prdm1^{gfp/gfp}* mice according to Flt3 and GFP expression (top) or sorted peritoneal B1 and splenic follicular B cells (Fo) (bottom). Data are presented as relative to *Hprt* with one sample arbitrarily set to one. Expression was analyzed in triplicate and data are the mean \pm SEM of a representative of three biological replicates.

(B) *Prdm1^{gfp/gfp}* B cells were cultured in LPS for 4 days, stained for Flt3, and sorted according to the quadrants depicted in Figure 5A (lower, left). Nuclear extracts from the equivalent of $1.5\text{--}2 \times 10^5$ sorted or freshly isolated resting B cells (rest) were subjected to an immunoblot with Pax5 and Blimp-1 antibodies.

(C) ChIP assay of Pax5 binding to the *Flt3* promoter. LPS-cultured splenic B cells were sorted as in (B) and the Flt3⁺ and Flt3⁻ fractions were subjected to ChIP \pm Pax5 antibody. 1:3-fold serial dilutions of the ChIP product was amplified with primers that span the Pax5-binding sites in the *Flt3* promoter. *Pax5^{-/-}* or wild-type pro-B cells were used as controls. Data are representative of two experiments.

Pax5-mediated gene repression occurred primarily post-translationally.

To examine whether the altered Pax5 activity seen in the Flt3⁺ B cell fraction from *Prdm1^{gfp/gfp}* mice was due to decreased or absent binding of Pax5 to DNA, we performed electro-mobility shift assays (EMSA) and chromatin immunoprecipitation assays (ChIP) on B cells sorted according

to Flt3 expression. However, neither EMSA (data not shown) nor ChIP (Figure 6C) demonstrated diminished binding of Pax5 to the *Flt3* promoter, suggesting that decreased occupancy of its binding sites is unlikely to explain the decreased Pax5 activity observed.

DISCUSSION

Loss of Blimp-1 function in mouse B cells has been studied previously via conditional mutagenesis (Shapiro-Shelef et al., 2003). In that model, *Prdm1* was specifically inactivated in B cells by Cre under the control of the *Cd19* promoter. Similarly to the data presented here, *Prdm1^{fl/fl}* *Cd19Cre* mice showed strongly reduced, but clearly detectable, amounts of serum Ig and a low Ig secretion in vitro (Shapiro-Shelef et al., 2003). This and the failure of these mice to mount an antigen-specific Ig response led to the conclusion that Blimp-1 is absolutely required for ASC differentiation (Shapiro-Shelef et al., 2003). However, the incomplete Cre-mediated deletion of the conditional allele in that study made it difficult to determine whether Blimp-1-independent differentiation pathways existed. The model for Blimp-1 deficiency presented here, via *Rag1^{-/-}* mice reconstituted with *Prdm1^{gfp/gfp}* fetal liver stem cells, has two principle advantages. First, because *Rag1^{-/-}* mice lack endogenous B cells and serum Ig, any detectable Ig must derive from the Blimp-1 mutant B cells. The second advantage of our mouse model is the ability to track Blimp-1 expression by means of GFP (Kallies et al., 2004, 2006), which enables us to detect cells with an active *Prdm1* locus on a single-cell basis in the presence or absence of functional Blimp-1 protein and to track defects in these B cells to a distinct developmental state.

By a number of criteria, we have shown here that *Prdm1^{gfp/gfp}* mice have low titers of serum Ig. This raised the question of whether this Ig was produced by a rare, fully functional, ASC population lacking Blimp-1 or was the result of low secretion by a Blimp-1-independent stage of ASC differentiation. An earlier study suggested that B1 B cells are capable of secreting Ig in the absence of Blimp-1 expression (Tumang et al., 2005). However, recent studies have shown that Ig secretion from B1 cells crucially depends on, and is paralleled by, Blimp-1 expression (Fairfax et al., 2007; Savitsky and Calame, 2006), indicating that full ASC differentiation from all B cell subsets depends on Blimp-1. Our data demonstrate the existence of a Blimp-1-independent phase of ASC differentiation, and we propose the term “preplasmablast” to describe this transient and low Ig-secreting stage of differentiation. We have not been able to detect any GFP⁺ B cells in the GCs of *Prdm1^{gfp/gfp}* mice, so it appears likely that, at least in the absence of Blimp-1, preplasmablasts arise either in post-GC cells or via an extrafollicular pathway. Interestingly, we observed a larger fraction of GFP⁺ B cells in Blimp-1-deficient mice than in controls, suggesting that preplasmablast cells accumulate because of their failure to proceed along the differentiation pathway. Alternatively, the large number of preplasmablasts in *Prdm1^{gfp/gfp}* mice could be

driven by homeostatic mechanisms resulting from the low titers of natural antibody as previously proposed (Agenes and Freitas, 1999).

In addition to Blimp-1, XBP-1 and IRF-4 are two transcription factors known to be essential for ASC generation (Mittrucker et al., 1997; Reimold et al., 2001). Whereas *Xbp1* is considered to be downstream of Blimp-1 (Shaffer et al., 2004), we show here that initial induction of *Xbp1* is largely the result of loss of Pax5-mediated suppression, and we propose that the subsequent high expression of *Xbp1* is a consequence of the UPR rather than being directly mediated by Blimp-1. In keeping with this, enforced expression of XBP-1 in *Prdm1*^{-/-} B cell cultures does not rescue ASC differentiation (Shapiro-Shelef et al., 2003). The relationship between Blimp-1 and IRF-4 is less clear. Although a recent study demonstrated that Blimp-1 expression occurs independently of IRF-4 (Klein et al., 2006), a subsequent report suggested that IRF-4 directly regulates *Prdm1* (Sciammas et al., 2006). Our observation, that high *Irf4* transcription did not occur in the absence of Blimp-1, supports the notion that *Irf4* is downstream of Blimp-1. Ectopically expressed IRF-4 was, however, not sufficient to complement the Blimp-1 deficiency. Interestingly, the ectopic expression of Blimp-1 in *Prdm1*^{gfp/gfp} B cells increased Ig secretion, but did not induce upregulation of *Irf4*. This finding suggests that Blimp-1 and IRF-4 are regulated independently from each other, a conclusion supported by the recent observation that cultured B cells from Vav-deficient mice show impaired Blimp-1 induction, but IRF-4 expression is unchanged (Stephenson et al., 2006). In summary, while initiation of ASC differentiation is Blimp-1 independent, completion of this process depends on the coordinate expression of all three factors, Blimp-1, IRF-4, and XBP-1.

A key question in the study of the transcriptional events that control ASC differentiation is how the stable long-term transcriptional program of B cells is silenced and replaced by the fundamentally different program that governs the plasma-cell fate. The current consensus is that this process is orchestrated by the direct repression of the B cell fate by Blimp-1 (Shapiro-Shelef and Calame, 2005). The data presented here, however, challenge this concept and provide a significant revision to this model by identifying a central role for the B cell maintenance-factor Pax5.

Mature B cells express Pax5 and ASC differentiation is paralleled by its downregulation (Delogu et al., 2006). Pax5 is known to regulate Ig production by repressing *Igh* (Linderson et al., 2004) and *Igk* (Roque et al., 1996) transcription as well as expression of *Xbp1* (Reimold et al., 1996) and *Igj* (Rinkenberger et al., 1996). Loss of Pax5 is sufficient to induce IgM secretion and Blimp-1 expression (Delogu et al., 2006; Nera et al., 2006). A limitation of previous studies examining ASC differentiation was that they were conducted on Blimp-1-sufficient B cells, thereby making it impossible to separate the stages in differentiation that were solely dependent on Pax5 repression from subsequent steps that were dependent on Blimp-1 induction. Microarray studies performed on wild-type and

Blimp-1-deficient B cells stimulated under conditions that support ASC differentiation (Shaffer et al., 2004) are again of limited utility in addressing this question, because the majority of cells in the cultures remain undifferentiated B cells and thus are not likely to identify Blimp-1-independent gene-regulatory processes occurring in a subset of cells.

By using the Blimp-1 GFP reporter, we show here that Pax5 is rapidly extinguished in Blimp-1⁺ cells, a finding compatible with Pax5 being a direct target of Blimp-1 repression (Lin et al., 2002). However, we further demonstrate that the early phase of Pax5 downregulation occurs independently of Blimp-1, because both GFP⁺ *Prdm1*^{gfp/gfp} and *Prdm1*^{+/gfp} preplasmablasts showed similar Pax5 downregulation and induction of Pax5-repressed genes, suggesting that other factors are involved in this process. In support of this conclusion, ectopic expression of Blimp-1 in some models did not silence Pax5, suggesting that repression of the Pax5 locus depends on factors other than Blimp-1 (Sciammas and Davis, 2004).

To further examine the role of Pax5 in initiating the transcriptional cascade leading to ASC, we took advantage of the recent comprehensive reports of Pax5-repressed genes (Delogu et al., 2006; Nera et al., 2006). Three molecules, Flt3, embigin, and PD-1, were particularly advantageous because they are cell-surface molecules whose expression is repressed by Pax5 upon B cell commitment, but reactivated during ASC differentiation. Examination of the expression kinetics of these proteins demonstrated that they were induced before, and thus in the absence of, Blimp-1. The simultaneous derepression of three independent Pax5 target genes provides compelling evidence that Pax5 activity is modulated prior to Blimp-1 induction in ASC differentiation. Importantly, we also found evidence for the Blimp-1-independent regulation of Pax5 activity by examining the expression of the Pax5-activated target genes *Cd23*, *Cd79a*, and *Blink* (Nutt et al., 1998; Schebesta et al., 2002; Visan et al., 2003). All three genes were distinctly downregulated in the Flt3⁺ fraction before the onset of Blimp-1 expression. These changes were, however, specific for individual genes: the expression of another Pax5-activated gene *Cd19* was found to be unchanged in Flt3⁺ B cells. Interestingly, we found that some in vivo B cell populations, including a large fraction of MZ and GC B cells, as well as B1 cells, resembled Flt3⁺ B cells in vitro in that they showed altered expression of Pax5 target genes. This poised gene-expression profile may explain the rapid kinetics by which both B1 and MZ B cells differentiate into ASC in vitro (Fairfax et al., 2007).

The suppression of Pax5 activity was independent of changes in its protein expression or in vivo DNA binding, raising the possibility that changes in the expression or activity of other transcription factors during B cell activation alter Pax5 activity. Indeed, it has been shown that Pax5 function is modulated by the relative abundance of Id2 (Gonda et al., 2003) and PU.1 (Linderson et al., 2004). We propose that a similar mechanism regulates the

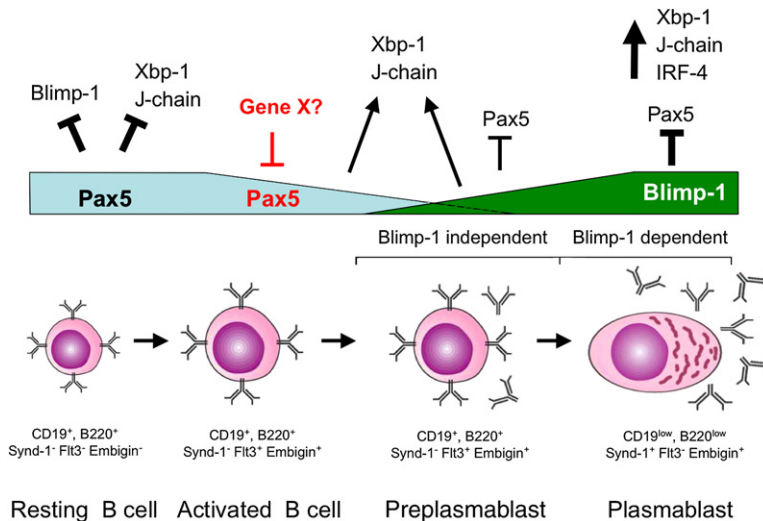


Figure 7. Model of ASC Differentiation

The stages of cellular differentiation from a resting B cell to an ASC and the associated markers are indicated. The relative Pax5 and Blimp-1 expression and function is indicated. \uparrow indicates positive influence on gene expression or function; \perp indicates repressive activity.

initiation of ASC differentiation, although the identity of the inhibitory factor operating on Pax5 is currently unknown.

Over the past few years a model of the transcriptional regulation of the terminal differentiation of B cells to the ASC fate has been proposed. The central point of this model is the mutually antagonistic interactions between a number of transcriptional repressors, including Pax5, BCL6, and Blimp-1 (Shapiro-Shelef and Calame, 2005). Although this model describes the delineation and transcriptional stability of the B cell and ASC states, it does not adequately explain how the mutual transcriptional repression is lifted to allow differentiation. The data presented here provide a number of insights into the earliest events in this process and allow us to extend the current model (Figure 7). We propose that in the resting state, B cells stably express Pax5, which represses the ASC program. Upon cell activation, Pax5 function is modified, through an unknown mechanism, altering the expression of a number of Pax5 target genes important for the ASC fate. We propose that this event initiates the ASC program and generates the preplasmablast stage, which is characterized by downregulation of Pax5, low Ig secretion, and Blimp-1 induction. Blimp-1 then silences Pax5 transcription, and thus the B cell program, by a classical feedback inhibition mechanism. Although the available evidence suggests that the preplasmablast is a transient step in normal differentiation, cells accumulate at this point in the absence of Blimp-1. This model provides a framework to investigate the interaction of Pax5 and Blimp-1 in controlling the B cell and ASC fates, respectively, as well as to investigate the roles played by other molecules, in particular OBF-1, Bach2, BCL6, and IRF-4, in the process.

EXPERIMENTAL PROCEDURES

Transplantation of Fetal Liver Cells

E14 embryos from intercrossed *Prdm1^{+/gfp}* (C57BL/6 Ly5.2) mice were genotyped and fetal liver chimeras generated in *Rag1^{-/-}* (C57BL/6 Ly5.1) recipients and analyzed after 8 weeks as described (Kallies et al., 2004). All animal experiments were conducted according to

the protocols approved by the Melbourne Health animal ethics committee.

Flow Cytometry

mAb against CD19 (1D3), B220 (RA3-6B2), Ly5.2 (ALI-4A2), Flt3 (A2F10.1), IgM (331.12), CD5 (Ly-1), and CD21 (7G6) were purified from hybridoma supernatant and conjugated in the author's laboratory. Anti-Synd-1 (281-2), PD-1 (J43), and CD23 (B3B4) were obtained from BD Biosciences, and peanut agglutinin (PNA) was from Vector Laboratories. The rat embigin mAb will be described elsewhere (S.L.N., unpublished). Cells were analyzed on an LSR and cell sorting was carried out on a DiVa flow cytometer (BD Biosciences).

ELISA and ELISpot Assay

Serum and supernatant Ig amounts were measured by ELISA as described previously (Hasbold et al., 2004). Mucosal IgA was isolated from fecal samples by the following procedure. 1 ml of 0.1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich) in PBS was added per 0.1 g of feces, then vortexed in a mini-bead beater for 10 s at 2500 rpm. Debris was removed by centrifugation and supernatants were assayed for IgA by ELISA. ELISpot assays were performed on MultiScreen-HA filter plates (Millipore). Cells were incubated for 4 hr at 37°C on precoated 96-well filter plates and developed with the appropriate enzyme-conjugated secondary antibodies and substrates.

Immunohistochemistry

Small intestines were harvested into OCT (Sakura Finetek) and frozen on dry ice. 5 μ m sections were cut onto polylysine-coated slides, fixed in acetone, rehydrated in PBS, and blocked with PBS/10% FCS before staining with anti-mouse IgA-HRP. Staining was visualized with diaminodenzidine (Sigma-Aldrich) and counterstained with haematoxylin.

In Vivo Induction of ASC

Mice were injected intravenously with 3 μ g *E. coli* LPS (O111:B4, Sigma-Aldrich) and analyzed at days 3 and 7. Immunization was with a single intraperitoneal injection of 100 μ g 4(hydroxy-3)-nitrophenyl (NP) coupled to keyhole limpet hemocyanin (KLH) in the ratio of 13:1, and IgG1 ASC activity in 500 sorted GFP⁺ populations was determined with NP-specific ELISpot as described (Smith et al., 1997).

In Vitro Cell Culture

Naive splenic B cells were purified by Percoll gradient centrifugation and B220 magnetic bead purification (Miltenyi Biotec) as described (Hasbold et al., 2004). Purified cells were cultured at 1×10^5 /ml with optimal concentrations of CD40 antibody (1C10, 10 μ g/ml), IL4

(500 U/ml, R&D Systems), and IL5 (2 ng/ml, R&D Systems). 4×10^5 cells/ml were used for LPS (20 μ g/ml) stimulation.

Western Blotting and Immunoprecipitation

The Pax5 mAb was generated in rats by immunization with a purified GST-Pax5 fusion protein consisting of amino acids 154–284 of mouse Pax5. mAbs were screened by immunoblotting via Pax5^{-/-} and wild-type pro-B cell lines (Nutt et al., 1998). Reactivity with an endogenous protein of the appropriate size for Pax5 (~50 kDa) protein was specifically detected for clone 1H9. The Blimp-1 mAb and immunoblotting was as previously described (Kallies et al., 2004). Ig κ -HRP and IgM-HRP were from Southern Biotechnology Associates. Serum IgG immunoprecipitation was carried out with protein G-sepharose. Protein extracts corresponding to equal cell numbers were loaded onto the gel, with equal protein loading confirmed with Ponceau Red stains of the membrane after protein transfer.

ChIP

For ChIP analysis, splenic B cells of *Prdm1*^{+gfp} mice were cultured with 20 μ g/ml LPS. After 3 days, GFP⁺ Flt3⁺ and GFP⁻ Flt3⁻ cells were sorted and fixed with 0.5% formaldehyde. 2.5×10^6 cells were used for each ChIP experiment as described (Holmes et al., 2006). 5×10^6 wild-type and Pax5^{-/-} pro-B cells (Nutt et al., 1998) were used as controls.

RT-PCR Analysis

In vitro cultured B cells were sorted and subjected to RT-PCR according to standard procedures. Primer sequences are as reported (Corcoran et al., 2005), except for the following: *Prdm1* 5'-AACACGTGGTCAACCCAAAG, 5'-AGGCTGCAGAGATGGATGTAG; *Blnk* 5'-CCCACCCAGAACCTGTC, 5'-TTCAGCCTCCTGGTCTGC; *Cd79a* 5'-TCTTCTTGTATACGCCTGTTTG, 5'-GATGTTAGACTGAAGGCTGAACC; *Irf4* 5'-GGACTACAATCGTGAGGAGGAC, 5'-ACGTCACAGGACATGATATGG. Detection of the spliced form of *Xbp1* was as described (Iwakoshi et al., 2003). Real-time PCR was carried out as described (Holmes et al., 2006) with the products being quantified in relation to *Hprt* amounts.

Retroviral Infection

The MSCV-IRF4-Puro and MSCV-Puro retroviral vectors were as reported (Tamura et al., 2005). Transfection of Phoenix cells and subsequent spin infection was performed as described (Corcoran et al., 2005). Purified splenic B cells were activated with anti-CD40+IL4+IL5 for 40 hr (as described above) and used for spin infections before being placed in the same medium. After overnight culture, transduced B cells were selected by puromycin (4 μ g/ml) for 48 hr. Surviving cells were then counted and analyzed by flow cytometry or recultured in media at 1×10^5 cells/ml overnight, and the supernatant was assayed for Ig.

Supplemental Data

One figure is available at <http://www.immunity.com/cgi/content/full/26/5/555/DC1/>.

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