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Defective B cell receptor-mediated responses in mice lacking the Ets protein, Spi-B

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Spi-B is a hematopoietic-specific Ets family transcription factor closely related to PU.1. Previous gene targeting experiments have shown that PU.1 is essential for the production of both lymphocytes and monocytes. We have now generated mice with a null mutation at the *Spi-B* locus. Unlike PU.1 mutant mice, *Spi-B*$^{-/-}$ mice are viable, fertile and possess mature B and T lymphocytes. However, *Spi-B*$^{-/-}$ mice exhibit severe abnormalities in B cell function and selective T cell-dependent humoral immune responses. First, although *Spi-B*$^{-/-}$ splenic B cells respond normally to lipopolysaccharide stimulation *in vitro*, these B cells proliferate poorly and die in response to B cell receptor (surface IgM) cross-linking. Secondly, *Spi-B*$^{-/-}$ mice display abnormal T-dependent antigenic responses *in vivo* and produce low levels of antigen-specific IgG_1, IgG_{2a} and IgG_{2b} after immunization. Finally, *Spi-B*$^{-/-}$ mice show a dramatic defect in germinal center formation and maintenance. In contrast to wild-type animals, germinal centers in *Spi-B*$^{-/-}$ mice are smaller and short-lived with significantly increased numbers of apoptotic B cells. Taken together, these results demonstrate that Spi-B is essential for antigen-dependent expansion of B cells, T-dependent immune responses and maturation of normal germinal centers *in vivo*.

Keywords: B cell antigen receptor/B lymphocytes/Ets factors/gene targeting/germinal centers

Introduction

Ets proteins regulate gene expression during a wide variety of developmental and adaptive cellular responses, including cellular differentiation, oncogenesis and T cell activation (Wasylyk *et al.*, 1993; Moreau-Gachelin, 1994; Muthusamy *et al.*, 1995). There are at least 18 different mammalian Ets proteins which display distinct but overlapping patterns of expression and bind to similar purine-rich DNA consensus elements (GGAA/T) (Wasylyk *et al.*, 1993; Moreau-Gachelin, 1994; Su *et al.*, 1996). Thus, it has been difficult to define precisely the specific functions of individual Ets proteins. The Ets protein Spi-B is most closely related to another Ets member, PU.1. PU.1 has been implicated as an important regulator of hematopoiesis by its expression pattern, putative target genes and role in erythroleukemic neoplasia (Moreau-Gachelin *et al.*, 1989; Schuetze *et al.*, 1993). PU.1 is highly expressed in B lymphocytic, granulocytic and monocytic cells (Klemsz *et al.*, 1990; Galson *et al.*, 1993; Hromas *et al.*, 1993; Chen *et al.*, 1995). Many genes which are important for B lymphoid and myeloid development have been identified as putative PU.1 target genes: Ig heavy chain (μ) (Nelsen *et al.*, 1993), Ig light chains (κ and λ) (Pongubala *et al.*, 1992; Eisenbeis *et al.*, 1993), mb-1 (Igα) (Feldhaus *et al.*, 1992; Hagman and Grosschedl, 1992), B29 (Igβ) (Omori and Wall, 1993), Ig J-chain (Shin and Koshland, 1993), G-CSF receptor (Smith *et al.*, 1994), M-CSF receptor (Zhang *et al.*, 1994), Fcγ receptor 1 (Perez *et al.*, 1994), Fcγ receptor IIIA (Feinman *et al.*, 1994), scavenger receptor (Moulton *et al.*, 1994), c-*fes* (Ray-Gallet *et al.*, 1995; Heydemann *et al.*, 1996), CD11b (Pahl *et al.*, 1992, 1993) and CD18 (Rosmarin *et al.*, 1992). Importantly, *PU.1*$^{-/-}$ mice have been generated via gene targeting (Scott *et al.*, 1994; McKercher *et al.*, 1996). *PU.1*$^{-/-}$ mice exhibit defects in the development of T lymphocytes, B lymphocytes, monocytes and granulocytes. Furthermore, the *PU.1*$^{-/-}$ mice generated by Scott *et al.* die at day 16.5 of gestation (Scott *et al.*, 1994).

Human Spi-B was first cloned from a Burkitt lymphoma library using the Ets domain of PU.1 as a probe (Ray *et al.*, 1992). Spi-B has a DNA-binding domain at its C-terminus and an acidic N-terminal domain which corresponds to the transactivation domain in PU.1. The human Spi-B protein shares 43% overall amino acid sequence identity and 67% DNA-binding domain sequence identity with human PU.1 (Ray *et al.*, 1992). Murine Spi-B and PU.1 share 70% sequence identity in their DNA-binding domain (Chen *et al.*, 1995). In addition to being structurally similar, PU.1 and Spi-B also bind to identical DNA-binding sequences and transactivate the same target genes *in vitro* (Ray-Gallet *et al.*, 1995; Su *et al.*, 1996). Spi-B is capable of binding to sites within all of the PU.1 target genes examined so far: SV40, c-*fes*, M-CSFR, FcγRI, β-globin, Ig J-chain, Igκ, CD11b and Epstein–Barr virus nuclear antigen 2 (Laux *et al.*, 1994; Chen *et al.*, 1995; Su *et al.*, 1996). Furthermore, Spi-B has been shown to transactivate the SV40 promoter (Ray *et al.*, 1992), Btk promoter (Muller *et al.*, 1997), M-CSFR promoter (Chen *et al.*, 1995), CD11b promoter (Chen *et al.*, 1995) and $Ig\lambda_{2\text{-}4}$ enhancer (Su *et al.*, 1996). Although Spi-B and PU.1 appear to share many target genes in erythroid, myeloid and lymphoid lineages by *in vitro* assays, Spi-B expression is restricted to the lymphoid lineages. In contrast to that which has been reported previously (Ray *et al.*, 1992), Spi-B protein is not found in myeloid cell lines and peripheral blood neutrophils (Chen *et al.*, 1995;

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Su *et al.*, 1996). Spi-B expression is restricted to and developmentally regulated in lymphocytes (Su *et al.*, 1996). Although its expression decreases in mature T cells, Spi-B expression increases as B cells mature, suggesting a critical role for Spi-B in differentiated B cells. Consistent with its lineage-specific expression, Spi-B mRNAs are restricted to the lymphoid organs of developing mouse embryos and adult mice (Su *et al.*, 1996).

Despite the similarities between Spi-B and PU.1, their distinct expression patterns suggest that they may play non-redundant roles *in vivo.* Furthermore, Spi-B cannot compensate for the absence of PU.1 in *PU.1*$^{-/-}$ mice which completely lack lymphoid and myeloid lineage development (Scott *et al.*, 1994; Olson *et al.*, 1995). To investigate the *in vivo* function of Spi-B, we generated mice with a null mutation at the *Spi-B* locus. In direct contrast to *PU.1*$^{-/-}$ animals, *Spi-B*$^{-/-}$ mice are viable, fertile and possess mature B and T cells. While the function of *Spi-B*$^{-/-}$ T cells appears to be normal, *Spi-B*$^{-/-}$ B cells exhibit severe abnormalities in functional and humoral responses. Although *Spi-B*$^{-/-}$ B cells proliferate normally in response to lipopolysaccharide (LPS) stimulation *in vitro*, they respond poorly to anti-IgM stimulation. Furthermore, *Spi-B*$^{-/-}$ mice exhibit defective T-dependent immune responses *in vivo*: they produce low levels of IgG_1, IgG_{2a} and IgG_{2b}, form small germinal centers which are not sustained over a 21 day period and display more apoptotic B cells in spleen sections as compared with wild-type animals. These B cell-specific defects are consistent with the restricted expression of Spi-B protein in the B lymphocytic lineage. Taken together, these data show that despite the presence of PU.1 in B cells, Spi-B plays a distinct role from PU.1 in B cell receptor-mediated expansion and responses.

Results

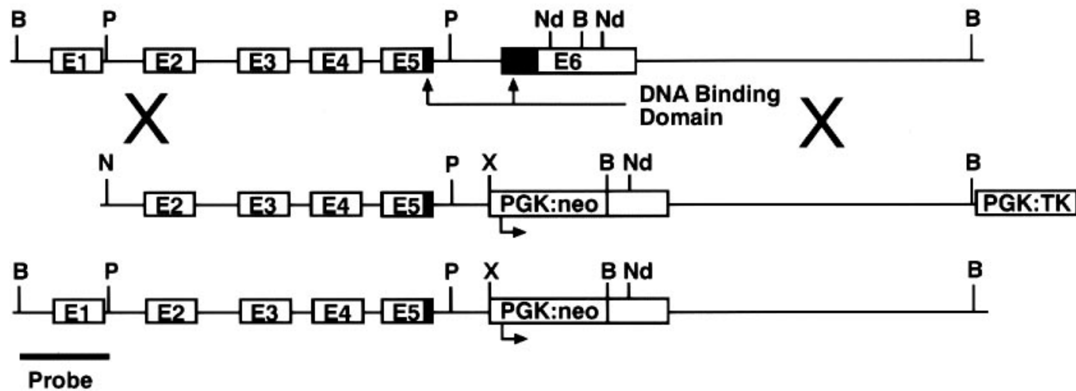
Spi-B is not essential for myeloid, erythroid or lymphoid development

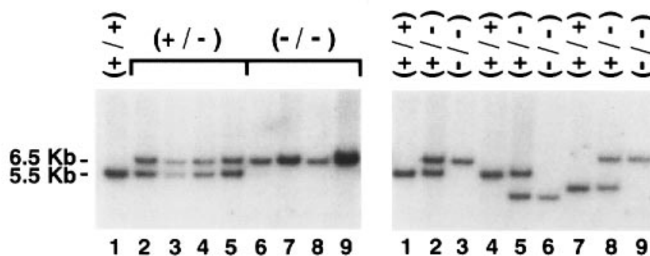
To investigate the function of Spi-B *in vivo*, we generated mice with a null mutation at the *Spi-B* locus. Following homologous recombination, the targeting vector deletes the DNA-binding domain of Spi-B and replaces it with the *PGK::neo* cassette (Figure 1A). Heterozygous (*Spi-B*$^{+/-}$) embryonic stem (ES) cells were obtained (Figure 1B, left panel, lanes 2–5) and used to generate *Spi-B*$^{-/-}$ ES cells via selection in high concentrations of G418 (Mortensen *et al.*, 1992) (Figure 1B, lanes 6–9). *Spi-B*$^{+/-}$ ES cells were injected into C57Bl/6 blastocysts, and chimeric mice gave rise to germline transmission of the targeted allele. Northern blots probed with either the Spi-B 3′-UTR or entire cDNA showed a complete absence of Spi-B mRNA in the spleens of homozygous mutant mice, confirming that the mutation produced a null allele (Figure 1C and data not shown). *Spi-B*$^{-/-}$ ES cells differentiated into erythrocytes, monocytes, megakaryocytes and granulocytes upon *in vitro* culture as embryoid bodies utilizing methylcellulose and appropriate cytokines (Figure 2) (Keller *et al.*, 1993; Olson *et al.*, 1995). All of the erythroid and myeloid genes examined by RT–PCR (β-globin, CD11b, CD18 and M-CSFR) were transcribed normally in four independent *Spi-B*$^{-/-}$ clones upon differentiation (data not shown).

Spi-B$^{-/-}$ mice were viable and fertile. Out of 261 progeny genotyped, 74 were wild-type (28%), 128 were heterozygous (49%) and 59 were homozygous mutant (23%). Four- to eight-week-old *Spi-B*$^{-/-}$ mice possessed normal numbers of splenocytes (*Spi-B*$^{+/+}$ $= 6.26 \pm 1.55 \times 10^7$, *Spi-B*$^{+/-}$ $= 8.59 \pm 2.28 \times 10^7$ and *Spi-B*$^{-/-}$ $= 5.67 \pm 1.42 \times 10^7$, $n = 7$) and thymocytes (*Spi-B*$^{+/+}$ $= 23.28 \pm 1.79 \times 10^7$, *Spi-B*$^{+/-}$ $= 27.24 \pm 2.4 \times 10^7$ and *Spi-B*$^{-/-}$ $= 19.72 \pm 1.36 \times 10^7$, $n = 5$). *Spi-B*$^{+/+}$, *Spi-B*$^{+/-}$ and *Spi-B*$^{-/-}$ embryonic hematopoietic tissues and adult thymi, spleens, lymph nodes and bone marrow were analyzed for cells expressing T cell surface antigens [CD2, CD3, CD4, CD8, CD25, Thy1.2, T cell receptor (TCR) αβ and TCRγδ], B cell antigens (CD43, B220, HSA, IgM, IgD and λ), macrophage surface proteins (CD11b and CD18) and granulocyte protein (Gr-1). No significant differences in antigen expression among *Spi-B*$^{+/+}$, *Spi-B*$^{+/-}$ and *Spi-B*$^{-/-}$ mice were noted. As shown in Figure 3, the profile of $CD4^+$ and $CD8^+$ thymocytes in *Spi-B*$^{-/-}$ mice was identical to that of wild-type animals. Furthermore, *Spi-B*$^{-/-}$ mice contained normal ratios of $CD4^+$ and $CD8^+$ T cells in the spleen. Importantly, the percentage (32–45%) and absolute number (*Spi-B*$^{+/+}$ $= 2.49 \pm 0.64 \times 10^7$, *Spi-B*$^{+/-}$ $= 3.35 \pm 0.78 \times 10^7$ and *Spi-B*$^{-/-}$ $= 2.39 \pm 1.08 \times 10^7$, $n = 5$) of $B220^+IgM^+$ splenic cells and IgM^+IgD^+ cells in *Spi-B*$^{-/-}$ mice were similar to *Spi-B*$^{+/+}$ and *Spi-B*$^{+/-}$ littermates. In addition, mutant animals expressed normal levels of surface CD40 antigen and B7.2 (data not shown). Therefore, Spi-B does not appear to play a critical, non-redundant role in B cell development.

Spi-B$^{-/-}$ *mice exhibit defective secondary responses to T-dependent antigen*

The mutagenesis of several B cell-restricted transcription factors such as Oct–2, NF-κB p50 and c-Rel leads to a profound reduction in all IgG subclasses and defective isotype switching (Corcoran and Karvelas, 1994; Kontgen *et al.*, 1995; Sha *et al.*, 1995). However, we determined that *Spi-B*$^{-/-}$ mice have normal basal levels of all Ig isotypes (not antigen-specific), including IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgA (Figure 4A). *Spi-B*$^{-/-}$ mice also rearranged and expressed their Igκ and λ loci on surface and secreted immunoglobulins (see Figure 4A). *Spi-B*$^{-/-}$ mice displayed a normal antigen-specific (DNP) humoral response when challenged with the T-independent antigen DNP–LPS. (Figure 4B). When *Spi-B*$^{-/-}$ mice were immunized with a T-dependent antigen [DNP–keyhole limpet hemocyanin (KLH)], their serum DNP-specific IgG_{2a} levels were significantly lower than those of *Spi-B*$^{+/+}$ littermates while IgM levels were 14-fold higher in the primary responses (Figure 4C). Otherwise, the primary response to DNP–KLH was normal. In marked contrast, secondary responses demonstrated a dramatic reduction in IgG_{2a} and IgG_{2b} levels (35-fold lower) and IgG_1 levels (3- to 5-fold lower) 8 days after reimmunization of *Spi-B*$^{-/-}$ mice (Figure 4D). This isotype profile suggests that *Spi-B*$^{-/-}$ B cells respond poorly to Th1 T cell signals even though normal levels of interferon-γ (IFN-γ) and interleukin-2 (IL-2) were produced and *Spi-B*$^{-/-}$ B cells expressed normal levels of receptors for both IFN-γ and IL-2 (data not shown). These data suggest that *Spi-B*$^{-/-}$ B cells exhibit a defect in class switching to the IgG_1, IgG_{2a}





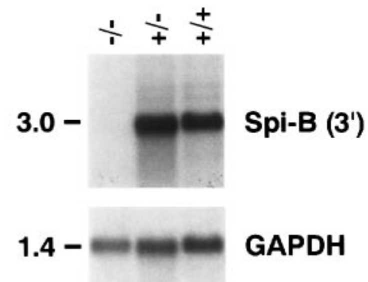


Fig. 1. Generation of Spi-B deficient mice. The wild-type allele, targeting vector and disrupted allele are shown (**A**). White boxes represent coding exons and black boxes symbolize the DNA-binding domain (only three amino acids of this domain reside in exon 5). Restriction enzyme sites used for cloning and Southern analysis are indicated as follows: B = *Bam*HI, Nd = *Nde*I, P = *Pst*I, X = *Xho*I. Southern blot analyses performed with *Bam*HI-digested genomic DNA were hybridized to the indicated 5′-probe (**B**, left panel, lanes 1–9). The wild-type allele generated a 5.5 kb *Bam*HI restriction fragment. Upon homologous recombination, ~0.8 kb of genomic Spi-B DNA is deleted and replaced by 1.8 kb of PGK::*neo*, making the recombined DNA fragment 1 kb larger than the wild-type. Additional restriction enzyme analyses with *Bam*HI and *Xho*I (B, right panel, lanes 4–6) or *Bam*HI and *Nde*I (right panel, lanes 7–9) confirmed the presence of targeted alleles. (**C**) Poly(A)$^+$ Northern blot analysis performed on *Spi-B*$^{-/-}$, *Spi-B*$^{+/-}$ and *Spi-B*$^{+/+}$ splenocytes. Probes containing the 3′-UTR (*Nde*I–*Bam*HI) or the entire cDNA of Spi-B detected no transcripts in the *Spi-B*$^{-/-}$ sample.

and IgG_{2b} isotypes upon secondary challenge. Alternatively, *Spi-B*$^{-/-}$ B cells expressing these IgG isotypes fail to expand upon reimmunization. Since reduced antibody production was not observed in non-antigen-specific or T-independent humoral responses, the defect in generating various IgG isotypes appears to be specific to T-dependent antigen stimulation in *Spi-B*$^{-/-}$ mice.

Spi-B$^{-/-}$ *mice fail to maintain their germinal centers following T-dependent antigenic challenge*

A natural consequence of a T-dependent immune response is the formation of germinal centers characterized by the presence of cells which bind the lectin peanut agglutinin (PNA$^+$ cells). Interestingly, the abnormal humoral immune responses to DNP–KLH were associated with abnormal germinal center formation in *Spi-B*$^{-/-}$ mice. PNA$^+$ germinal centers generated in the spleens of *Spi-B*$^{-/-}$ mice 10 days after DNP–KLH immunization were consistently smaller than those in *Spi-B*$^{+/+}$ animals (Figure 5A and B). Furthermore, unlike wild-type animals, *Spi-B*$^{-/-}$ mice failed to maintain the germinal centers for the 28 day period tested (Figures 5C and D, and 6E and F). In fact, *Spi-B*$^{-/-}$ germinal centers were mostly eliminated as early as 21 days after antigenic challenge (data not shown). B220 immunostaining revealed that primary follicles were present in both *Spi-B*$^{+/+}$ and *Spi-B*$^{-/-}$ mice (Figure 6C and D). However, *Spi-B*$^{-/-}$ spleens contained more B220$^+$ B cells outside of the circumscribed white pulp, indicating a deterioration of the follicular organization. In addition, hematoxylin and eosin (H&E) staining indicated that the follicular zonation separating the germinal center, mantle zone and marginal zone was abnormal in *Spi-B*$^{-/-}$ mice as compared with wild-type animals (Figure 6A and B). This lack of zonation was consistently observed by day 21 post-immunization in six animals studied. Half of the *Spi-B*$^{-/-}$ mice sacrificed 10 days after injection also showed early deterioration of zonation (data not shown). None of their wild-type littermates exhibited this germinal center phenotype. PNA and H&E

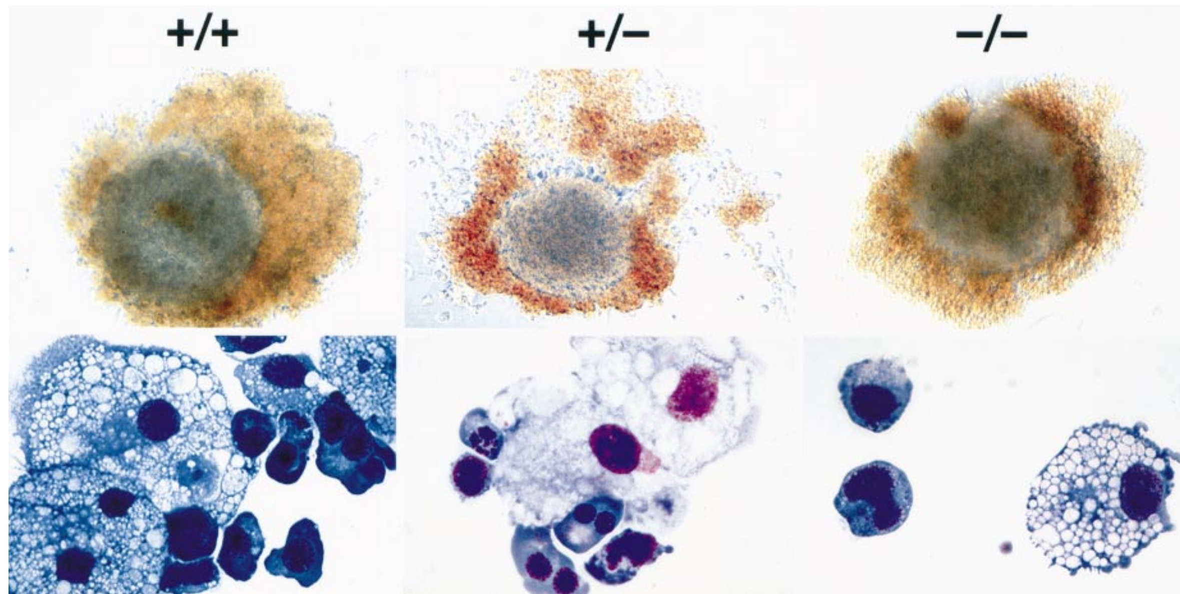


Fig. 2. *In vitro* differentiation of *Spi-B*$^{+/+}$, *Spi-B*$^{+/-}$ and *Spi-B*$^{-/-}$ ES cells. The genotypes of the ES cells are indicated. Top panels depict differentiated 'embryoid bodies' on day 11 of culture; bottom panels show erythrocytes and macrophages by May–Grunwald–Giemsa staining. Magnification: top, 20×; bottom, 100×.

staining of splenic sections from *Spi-B*$^{-/-}$ mice undergoing secondary immunization also displayed a similar phenotype with early disappearance of PNA$^+$ cells and follicular zonation (data not shown).

B cell receptor-mediated survival is defective in vivo in the absence of Spi-B

The early elimination of PNA$^+$ cells and follicular zonation suggests that Spi-B may be important for B cell expansion or survival. To test the latter hypothesis, immunostaining with TdT-mediated dUTP-biotin nick end labeling (TUNEL) was performed on splenic sections of DNP–KLH-challenged mice. A TUNEL-positive reaction was indicated by the accumulation of a blue/black reaction product within the nucleus of the cell. Immunostaining with TUNEL revealed that upon antigenic challenge, *Spi-B*$^{-/-}$ spleens contained on average six times as many apoptotic cells as wild-type littermates (Figure 7A and B). Many microscopic fields from stained sections of *Spi-B*$^{+/+}$ and *Spi-B*$^{-/-}$ spleens were quantitated for TUNEL$^+$ cells. *Spi-B*$^{+/+}$ splenic sections contained 13.2 (± 1.8 standard error) TUNEL$^+$ cells per field while *Spi-B*$^{-/-}$ sections contained 78.4 (± 6.4 standard error) TUNEL$^+$ cells. Moreover, *Spi-B*$^{-/-}$ spleens obtained from immunized animals contained five times as many TUNEL$^+$ cells as those from non-immunized *Spi-B*$^{-/-}$ animals. Importantly, TUNEL$^+$ cells were shown to be B220$^+$, confirming that the apoptotic cells are B cells (Figure 7C). In these TUNEL/B220 assays, TUNEL$^+$ B220$^+$ cells stain simultaneously brownish (B220) and bright red (TUNEL). In direct contrast, TUNEL staining with the T lymphoid marker CD5 demonstrated that apoptotic cells were not T cells (Figure 7D). CD5 does stain the B-1 subpopulation of B cells, but these cells are extremely rare in the spleen. Furthermore, the TUNEL$^+$ cells are amongst a cluster of B220$^+$ B cells and are clearly separate from the cluster of CD5$^+$ T cells. Taken together, these results suggested a model in which *Spi-B*$^{-/-}$ B cells fail to survive upon

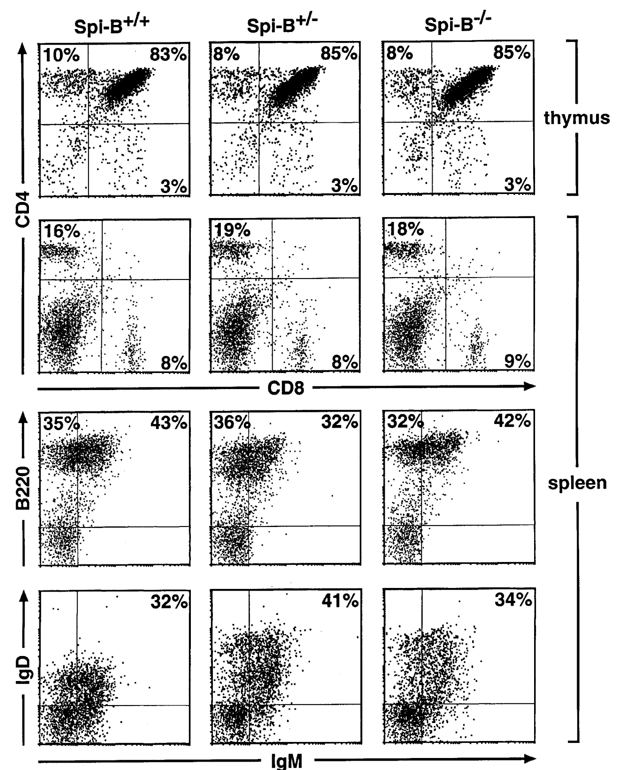


Fig. 3. *Spi-B*$^{-/-}$ mice have mature T and B lymphocytes. Representative flow cytometry analysis of adult *Spi-B*$^{+/+}$, *Spi-B*$^{+/-}$ and *Spi-B*$^{-/-}$ thymocytes and splenocytes showing no difference in CD4/CD8, B220/IgM or IgD/IgM immunostaining.

antigen stimulation via the B cell antigen receptor (BCR). The abnormal apoptosis of *Spi-B*$^{-/-}$ B cells within the germinal centers may lead to their early elimination and a subsequent diminution in levels of serum antibody.

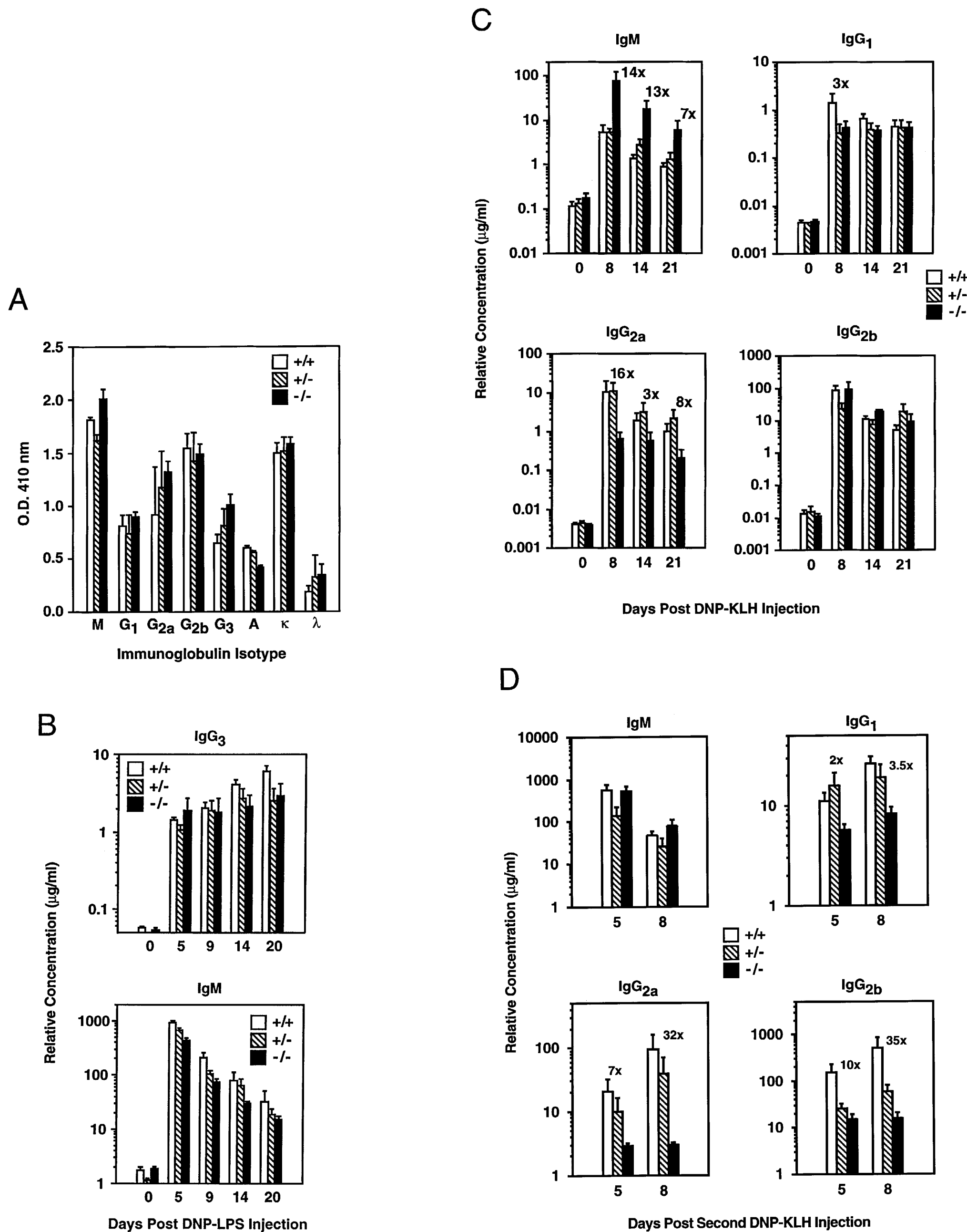


Fig. 4. Analysis of T-independent and T-dependent responses in *Spi-B*$^{+/+}$, *Spi-B*$^{+/–}$ and *Spi-B*$^{–/–}$ animals. *Spi-B*$^{+/+}$ cells are represented by white bars, *Spi-B*$^{+/–}$ by striped bars and *Spi-B*$^{–/–}$ cells by black bars. Levels of total immunoglobulin isotypes in the serum of unchallenged mice (n = 3 for each genotype) (**A**), DNP-specific antibody responses in DNP–LPS-challenged mice (n = 3 for each genotype) (**B**), DNP–KLH-immunized mice (n = 8) (**C**), or DNP–KLH-reimmunized mice (n = 4) (**D**) are shown as determined by ELISA. Data are represented as the geometric mean and standard error.

Spi-B$^{–/–}$ *B cells respond poorly to IgM stimulation, but proliferate normally during LPS treatment in vitro*

To examine this model further, we studied the proliferative responses of purified *Spi-B*$^{–/–}$ B and T cells *in vitro*. Purified *Spi-B*$^{–/–}$ splenic and lymphatic T cells proliferated normally in response to anti-CD3, PMA + ionomycin or concanavalin A (ConA) stimulation *in vitro* (Figure 8A, right panel). *Spi-B*$^{–/–}$ B cells also responded normally to the polyclonal mitogen LPS (Figure 8A, left panel). However, as shown in Figure 8A, the proliferation of purified *Spi-B*$^{–/–}$ splenic B cells in response to IgM cross-linking was reduced by 67% ($P<0.0002$) as compared with wild-type cells. This proliferative defect persisted even when saturating levels of anti-IgM were added (data not shown). Suboptimal amounts of anti-IgM produced a 6- to 7-fold reduction ($P<10^{-6}$) in *Spi-B*$^{–/–}$ B cell proliferation (Figure 8A, middle panel). Interestingly, *Spi-B*$^{+/–}$ B cells exhibited an intermediate phenotype ($P<0.015$). *Spi-B*$^{–/–}$ B cells did respond to CD40, IL-4 and IL-6 costimulation. However, the overall response remained ~33% of normal (Figure 8A, middle panel and data not shown). *Spi-B*$^{–/–}$ B cells also proliferated normally in response to PMA + ionomycin stimulation (data not shown). These results suggest that mutant B cells possess intact CD40, IL-4, IL-6 and protein kinase C (PKC) signaling pathways. We

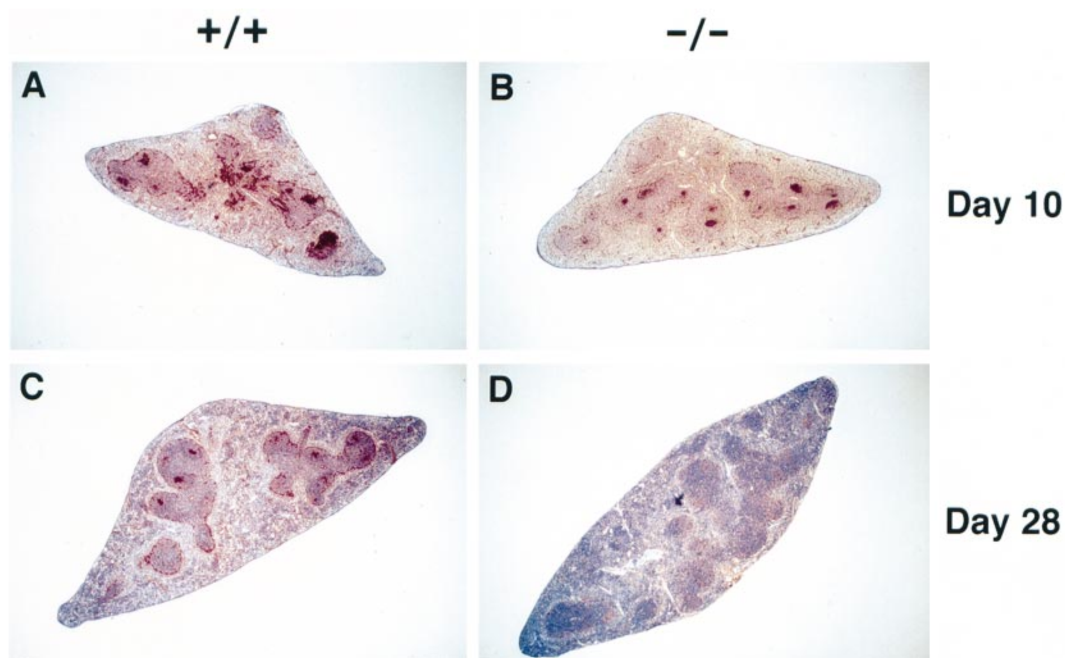


Fig. 5. *Spi-B*$^{-/-}$ mice are capable of forming but not sustaining their germinal centers. *Spi-B*$^{+/+}$ and *Spi-B*$^{-/-}$ mice were sacrificed on day 10 (**A** and **B**) or day 28 (**C** and **D**) post-DNP–KLH immunization. Their spleen sections were processed and stained with peanut agglutinin antigen (PNA). PNA^{+} cells appear dark brown in these assays.

conclude that *Spi-B*$^{-/-}$ B cells exhibited a dramatic and specific defect in their ability to expand in response to stimulation through the BCR. Furthermore, we can say with confidence that a critical Spi-B target(s) lies between the surface BCR and PKC.

Upon IgM stimulation, Spi-B$^{-/-}$ B cells become activated and enter the cell cycle, but die in vitro

There are several possible steps within BCR signaling and proliferation pathways where Spi-B may play an indispensable role. To examine further the role of Spi-B in BCR-mediated proliferation, classical B cell activation responses were investigated. An increase in surface expression of proteins such as MHC class II, CD19, CD44 and CD69 has been associated with B cell activation (Testi *et al.*, 1989; Carter and Fearon, 1992; Swat *et al.*, 1993). *Spi-B*$^{-/-}$ B cells up-regulated these activation markers normally 24 h after IgM stimulation and the expression of each protein was sustained over a 72 h time period in the *Spi-B*$^{+/+}$, *Spi-B*$^{+/-}$ and *Spi-B*$^{-/-}$ cells (Figure 8B and data not shown). Many genes known to be involved in BCR signaling pathways are putative target genes of Ets proteins. These include Btk (Sideras *et al.*, 1994), Blk (Lin *et al.*, 1995), mb-1 (Hagman and Grosschedl, 1992) and B29 (Omori and Wall, 1993). *Spi-B*$^{-/-}$ splenic B cells expressed normal mRNA levels of Btk, Blk, mb-1 and B29 as well as the syk, fyn and lyn tyrosine kinases (data not shown). It is of note that, VAV was also present at normal levels in *Spi-B*$^{-/-}$ B cells, even though *Vav*$^{-/-}$ B cells have a proliferative defect similar to that of *Spi-B*$^{-/-}$ B cells (Fischer *et al.*, 1995; Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995). Finally, levels of PU.1 mRNA production were identical in *Spi-B*$^{+/+}$, *Spi-B*$^{+/-}$ and *Spi-B*$^{-/-}$ B cells (data not shown). Taken together, these data indicate that Spi-B is not essential for B cells to up-regulate their activation markers or to transcribe multiple components of the BCR signaling pathway. However, Spi-B is critical for B cell expansion in response to BCR-mediated proliferation signals.

Lastly, propidium iodide- (PI) based cell cycle analysis revealed that *Spi-B*$^{-/-}$ B cells cultured with anti-IgM antibodies were able to enter the S phase of the cell cycle at the same rate and ratio as *Spi-B*$^{+/+}$ and *Spi-B*$^{+/-}$ cells (Figure 8C). Notably, the G_1 peak of IgM-stimulated *Spi-B*$^{-/-}$ B cells diminished over the 72 h time period and gave rise to a higher percentage of the non-viable population (Figure 8C). Although a comparable percentage of *Spi-B*$^{-/-}$ B cells were cycling in comparison with the wild-type control, the number of live cells at each time point was lower in the mutant culture. This is particularly clear 72 h after anti-IgM stimulation. Thus, *Spi-B*$^{-/-}$ B cells activated by anti-IgM were able to enter the cell cycle, even though the possibility remains that they are unable to complete the cell cycle. Consistent with previous observations, LPS-stimulated *Spi-B*$^{-/-}$ B cells produced a comparable cell cycle profile to wild-type cells throughout a 72 h time period (data not shown).

Since *Spi-B*$^{-/-}$ B cells were able to become activated and proliferate, the low [^{3}H]thymidine incorporation could only then be explained by inappropriate cell death upon IgM stimulation. The *in vitro* culture of B cells requires stimulation by anti-IgM cross-linking, LPS or other mitogens for cell survival. A table on cell viability was compiled by trypan-blue, PI, 7-AAD or annexin V exclusion. All of the procedures (trypan-blue, PI, 7-AAD and annexin V) gave the same results. As shown in Table I, 3- to 4-fold fewer cells survived in IgM-stimulated *Spi-B*$^{-/-}$ cultures as compared with wild-type. The number of live *Spi-B*$^{-/-}$ B cells in culture 72 h after IgM stimulation correlated perfectly to the reduced levels of [^{3}H]thymidine incorporation (67%) (Figure 8A). Furthermore, *Spi-B*$^{+/-}$ B cells exhibited a 50% decrease in viability in comparison

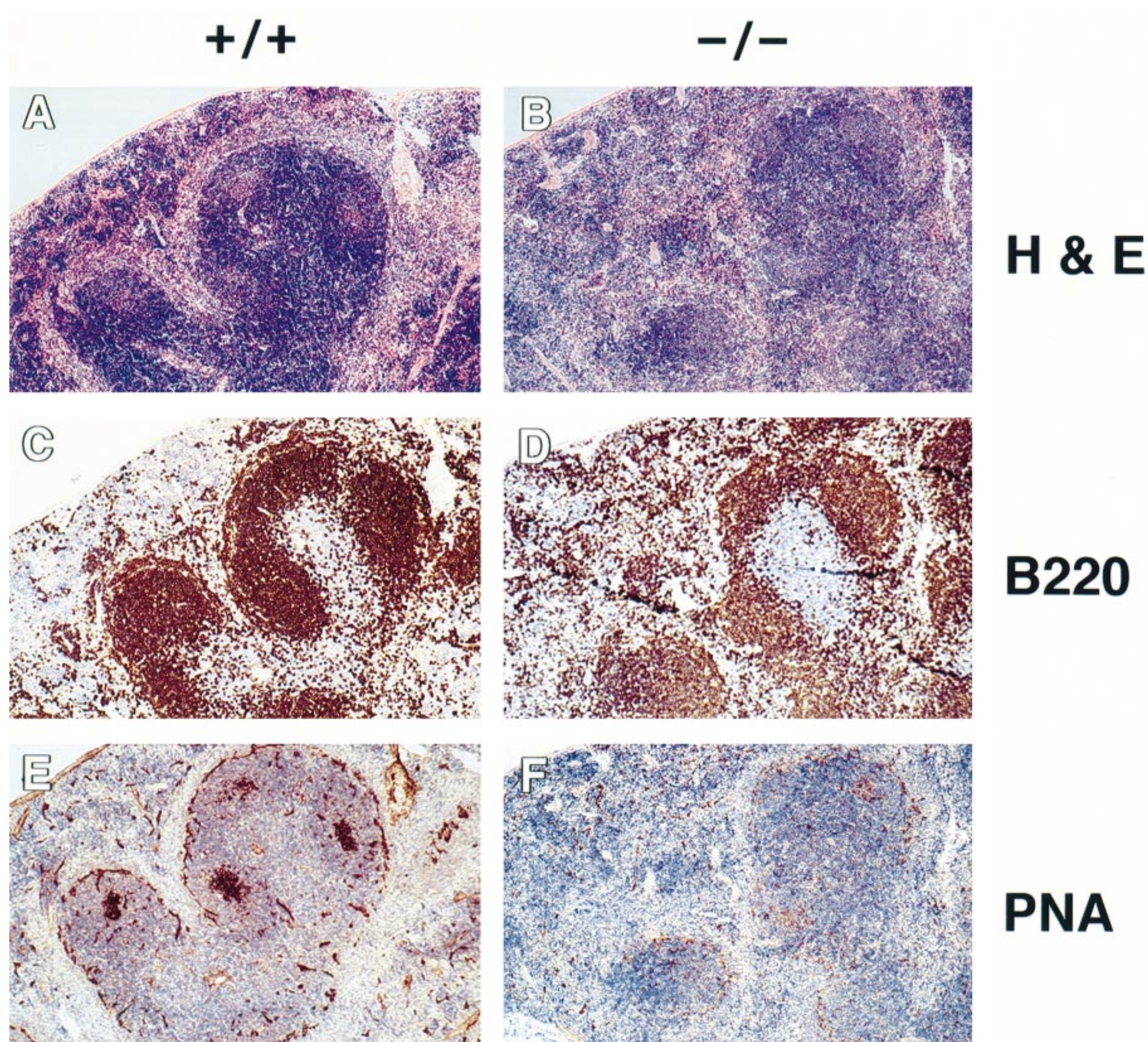


Fig. 6. Analysis of germinal centers in *Spi-B*$^{+/+}$ and *Spi-B*$^{-/-}$ mice upon T-dependent antigenic challenge. Spleen sections of *Spi-B*$^{+/+}$ (**A**, **C** and **E**) and *Spi-B*$^{-/-}$ (**B**, **D** and **F**) mice at day 28 post-DNP–KLH injection stained with hematoxylin and eosin (A and B), anti-B220 antibody (C and D) or lectin peanut agglutinin antigen (E and F). Magnification in (A–F): 10×.

with *Spi-B*$^{+/+}$ cells. Importantly, this survival defect was only observed in anti-IgM-treated cells. The viability of either unstimulated (5.6%) or LPS-treated (34.8%) *Spi-B*$^{-/-}$ B cells was identical to that of *Spi-B*$^{+/+}$ and *Spi-B*$^{+/-}$ cells (Table I). Therefore, the ability of anti-IgM cross-linking to protect *in vitro* cultured cells from apoptosis is significantly reduced in *Spi-B*$^{-/-}$ cells. The observation that *Spi-B*$^{-/-}$ B cells exhibited a 3- to 4-fold decrease in viability *in vitro* is similar to the increased apoptosis in the T-dependent antigen-treated *Spi-B*$^{-/-}$ mice *in vivo*.

Discussion

In these studies, we have clearly established that Spi-B is indispensable for BCR-mediated responses *in vivo* and *in vitro*. Unlike PU.1, Spi-B does not appear to play an essential role in myeloid and lymphoid development. From expression studies we have established that Spi-B mRNA and protein are not detected in myeloid cells (Chen *et al.*, 1995; Su *et al.*, 1996). Thus it is perhaps not surprising that mature macrophages and granulocytes developed normally in *Spi-B*$^{-/-}$ mice and *in vitro* differentiated *Spi-B*$^{-/-}$ ES cells, unless Spi-B is also expressed in early hematopoietic progenitors. While it might also play a role in earlier B cell development, its function in B cell maturation may be masked by the presence of PU.1 in the B cells of *Spi-B*$^{-/-}$ mice. There are precedents where structurally similar transcription factors share the same target genes and functionally substitute for one another. For example, Myf-5 and MyoD have redundant roles in myogenesis. While *Myf-5*$^{-/-}$ or *MyoD*$^{-/-}$ mice possess normal skeletal muscle (Braun *et al.*, 1992; Rudnicki *et al.*, 1992), *Myf-5*$^{-/-}$*MyoD*$^{-/-}$ mice completely lack myogenesis (Rudnicki *et al.*, 1993). Similarly, En-1 and En-2 are two structurally similar proteins with different *in vivo* functions; however, when En-2 is 'knocked in' under the control of the En-1 promoter, it rescues *En-1*$^{-/-}$ embryos from embryonic lethality (Hanks *et al.*, 1995). This demonstrates that the primary difference between En-1 and En-2 is their divergent expression patterns. *Spi-B*$^{-/-}$ B cells mature, survive and experience isotype switching, but they cannot respond normally to anti-IgM stimulation *in vitro* and T-dependent antigenic challenge *in vivo*. Therefore, Spi-B plays a unique role (apart from PU.1) in B cell function.

Isotype switching is a complex process involving

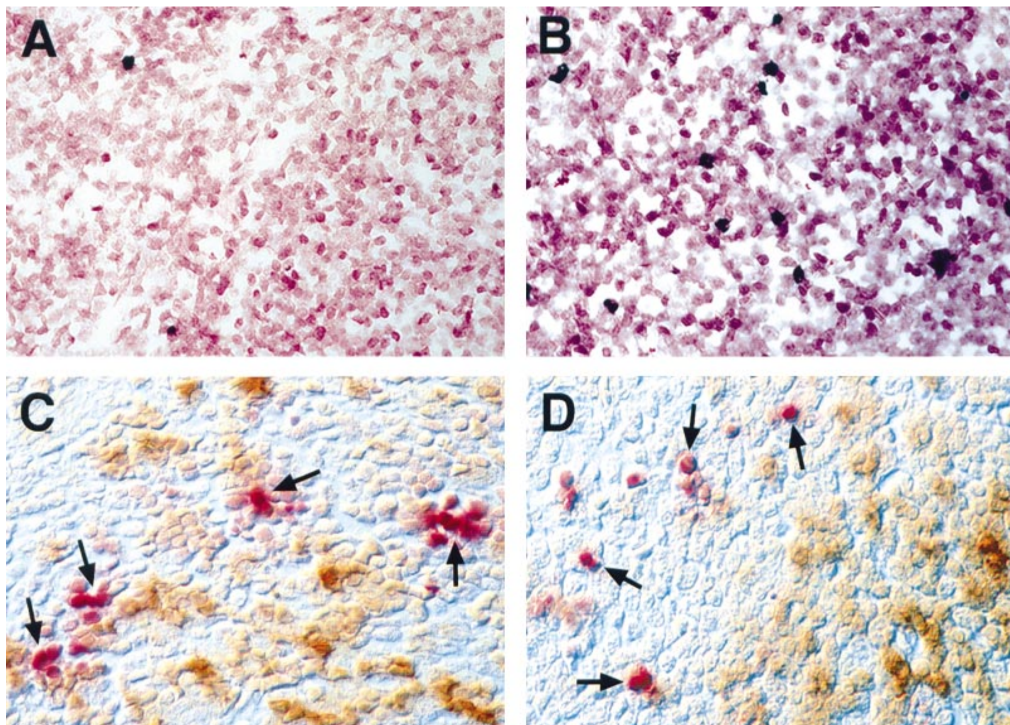


Fig. 7. Analysis of programed cell death in immunized *Spi-B*$^{+/+}$ and *Spi-B*$^{–/–}$ mice. Spleen sections of *Spi-B*$^{+/+}$ (**A**) and *Spi-B*$^{–/–}$ (**B**) mice 28 days after DNP–KLH injection assayed for apoptosis by TUNEL immunohistochemistry. TUNEL^{+} cells appear blue/black in this assay. Spleen sections of *Spi-B*$^{–/–}$ mice stained with TUNEL plus either B220 (**C**) or CD5 (**D**). B220^{+} or CD5^{+} cells appear dark brown while TUNEL^{+} cells appear bright red. TUNEL^{+}B220^{+} cells in (C) appear brownish red while the TUNEL^{+}CD5$^{–}$ cells in (D) appear red only. Magnification in (A) and (B): 20×. Magnification in (C) and (D): 40×.

multiple components which can affect the outcome in different ways. The type of antigens exposed to B cells, cytokines secreted by T cells and costimulation presented to B cells are known to influence isotype switching. In this study we have shown that *Spi-B*$^{–/–}$ B cells exhibit antigen-specific defects in cell expansion and antibody production, but respond normally to costimulation. The reduction in antigen-specific IgG_{2a} and IgG_{2b} isotypes suggests that *Spi-B*$^{–/–}$ B cells might not respond efficiently to Th1-type cytokines, even though IFN-γ and IL-2 levels were equal in *Spi-B*$^{+/+}$, *Spi-B*$^{+/–}$ and *Spi-B*$^{–/–}$ mice. Furthermore, *Spi-B*$^{–/–}$ B cells responded to IL-4 and IL-6, but neither was sufficient to compensate for the poor responses of *Spi-B*$^{–/–}$ B cells to anti-IgM treatment *in vitro.* These data suggest that the IFN-γ-responsive elements of germline IgG_{2a} and IgG_{2b} promoters may include binding sites for Spi-B. Alternatively, since no target genes have been identified for Spi-B and none of the putative PU.1 target genes is directly involved in isotype switching, the reduced levels of antigen-specific IgG_{2a}, IgG_{2b} and IgG_1 produced by *Spi-B*$^{–/–}$ mice can also be a consequence of the failure of cell expansion and early deterioration of germinal centers. Increased apoptosis of splenic B cells in *Spi-B*$^{–/–}$ mice after immunization favors the latter hypothesis.

The inability of *Spi-B*$^{–/–}$ germinal centers to be maintained represents an unprecedented consequence of the Spi-B mutation. Recent mutagenesis of genes encoding CD40, CD40 ligand, CD19, lyn, lymphotoxin-α, BCL-6, CR2 and the B cell transcription coactivator OCA-B have resulted in a failure of germinal center formation in deficient mice (Castigli *et al.*, 1994; Xu *et al.*, 1994; Hibbs *et al.*, 1995; Ahearn *et al.*, 1996; Kim *et al.*, 1996; Matsumoto *et al.*, 1996; Schubart *et al.*, 1996; Dent *et al.*, 1997). These findings suggest that such mice are unable to develop memory B cells due to their respective germinal center defects (Xu *et al.*, 1994). In marked contrast, *Spi-B*$^{–/–}$ mice initiate the production of germinal centers within splenic primary B cell follicles, but these structures decay prematurely due to BCR-mediated apoptosis. This raises the possibility that the memory B cell population is not maintained in *Spi-B*$^{–/–}$ mice. However, it is no longer certain that germinal centers are required for either B cell memory or BCR affinity maturation by somatic hypermutation (Matsumoto *et al.*, 1996). Thus, it will be important to examine the status of both somatic mutations and memory cells in *Spi-B* mice. The inability of *Spi-B*$^{–/–}$ B cells to undergo somatic mutation and affinity maturation may explain the increased apoptosis in the spleens of *Spi-B*$^{–/–}$ mice.

From *in vitro* assays we have shown that the inability of *Spi-B*$^{–/–}$ B cells to survive upon stimulation is BCR-specific. The proliferative defect observed in BCR-stimulated *Spi-B*$^{–/–}$ B cells is reminiscent of the survival defect reported for activated *Ets-1*$^{–/–}$ T cells (Muthusamy *et al.*, 1995) and multipotent hematopoietic progenitors in *PU.1*$^{–/–}$ mice (Scott *et al.*, 1997). It is tempting to speculate that Ets proteins are required for the normal survival of many blood cell types. Similarly to BCR-stimulated *Spi-B*$^{–/–}$ B cells, *Ets-1*$^{–/–}$ T cells apoptosed upon TCR-mediated stimulation but proliferated normally during PMA + ionomycin treatment (Muthusamy *et al.*, 1995; J.M.Leiden, personal communication,). Furthermore, Ets-1 has been shown to become phosphorylated by calcium/calmodulin-

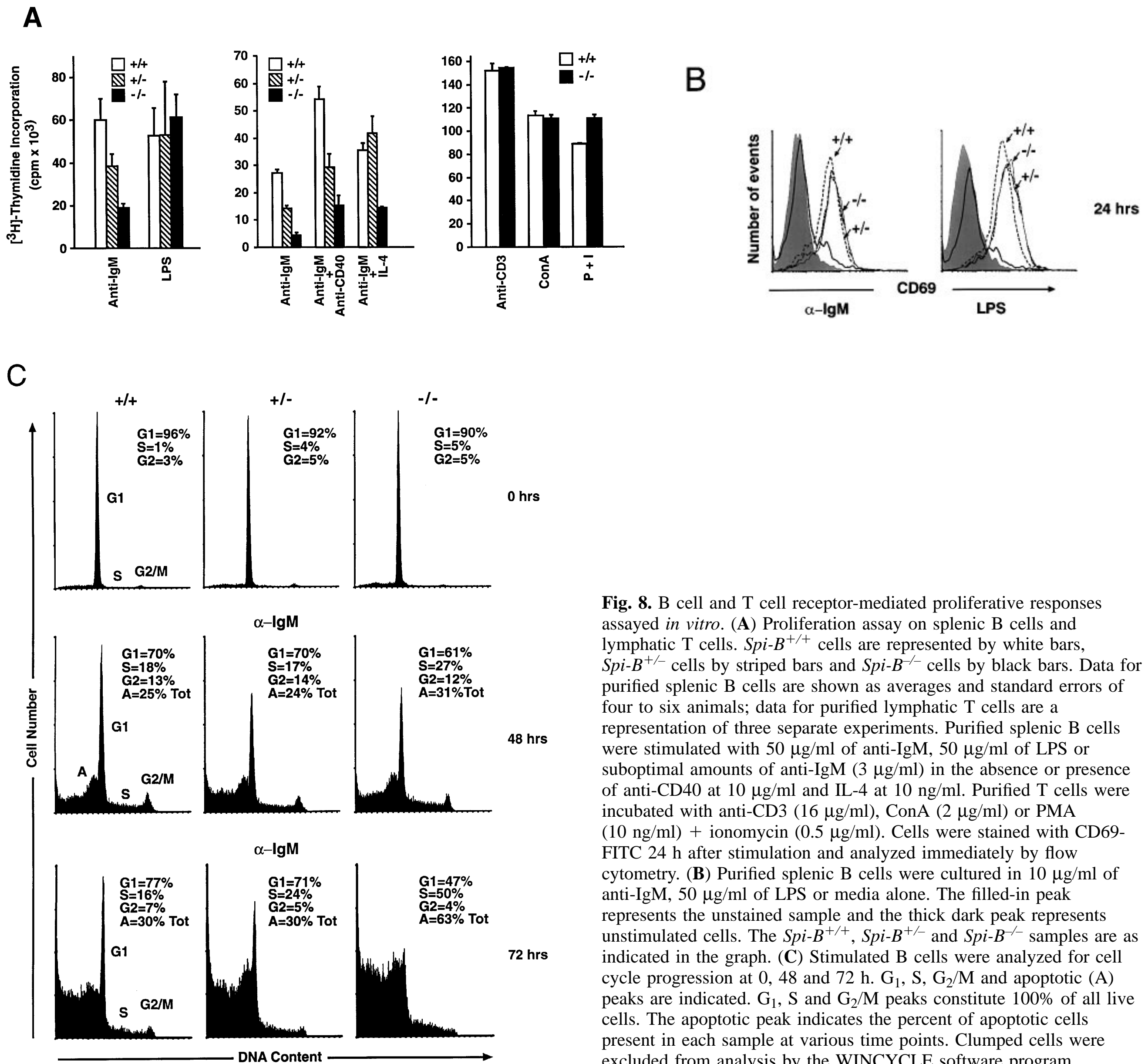


Fig. 8. B cell and T cell receptor-mediated proliferative responses assayed *in vitro*. (**A**) Proliferation assay on splenic B cells and lymphatic T cells. *Spi-B*$^{+/+}$ cells are represented by white bars, *Spi-B*$^{+/–}$ cells by striped bars and *Spi-B*$^{–/–}$ cells by black bars. Data for purified splenic B cells are shown as averages and standard errors of four to six animals; data for purified lymphatic T cells are a representation of three separate experiments. Purified splenic B cells were stimulated with 50 µg/ml of anti-IgM, 50 µg/ml of LPS or suboptimal amounts of anti-IgM (3 µg/ml) in the absence or presence of anti-CD40 at 10 µg/ml and IL-4 at 10 ng/ml. Purified T cells were incubated with anti-CD3 (16 µg/ml), ConA (2 µg/ml) or PMA (10 ng/ml) + ionomycin (0.5 µg/ml). Cells were stained with CD69-FITC 24 h after stimulation and analyzed immediately by flow cytometry. (**B**) Purified splenic B cells were cultured in 10 µg/ml of anti-IgM, 50 µg/ml of LPS or media alone. The filled-in peak represents the unstained sample and the thick dark peak represents unstimulated cells. The *Spi-B*$^{+/+}$, *Spi-B*$^{+/–}$ and *Spi-B*$^{–/–}$ samples are as indicated in the graph. (**C**) Stimulated B cells were analyzed for cell cycle progression at 0, 48 and 72 h. G_1, S, G_2/M and apoptotic (A) peaks are indicated. G_1, S and G_2/M peaks constitute 100% of all live cells. The apoptotic peak indicates the percent of apoptotic cells present in each sample at various time points. Clumped cells were excluded from analysis by the WINCYCLE software program.

dependent kinases upon anti-IgM cross-linking (Valentine *et al.*, 1995). This phosphorylation is PKC independent. We postulate that Spi-B regulates components of the BCR signaling pathway causing *Spi-B*$^{–/–}$ B cells to respond poorly to anti-IgM stimulation. *Spi-B*$^{–/–}$ B cells proliferate normally in response to PMA + ionomycin, suggesting that the PKC signaling pathway does not involve Spi-B-regulated genes.

Bcl-2 family members have been shown to regulate B cell survival (Oltvai *et al.*, 1993; Veis *et al.*, 1993; Motoyama *et al.*, 1995). In addition, Bcl-x_L expression increases upon surface IgM cross-linking, CD40 signaling or LPS stimulation, while Bcl-2 expression stays the same (Grillot *et al.*, 1996). Importantly, the protein expression of Bcl-2, Bcl-x_L and Bax was normal in anti-IgM- or anti-IgM + anti-CD40-stimulated *Spi-B*$^{–/–}$ B cells (data not shown). The BCR-mediated proliferative defect exhibited by *Spi-B*$^{–/–}$ B cells is reminiscent of those reported for immature B cells and tolerant B cells. Like immature or tolerant B cells, *Spi-B*$^{–/–}$ B cells undergo apoptosis when

Table I. The viability of *Spi-B*$^{+/+}$, *Spi-B*$^{+/–}$ and *Spi-B*$^{–/–}$ B cells *in vitro*

		% survival		
		24 h	48 h	72 h
None	+/+	37.1 ± 2.9	20.8 ± 2.8	5.2 ± 1.0
	+/–	38.2 ± 4.3	21.5 ± 4.4	6.1 ± 0.8
	–/–	34.9 ± 2.9	17.1 ± 2.2	5.6 ± 1.1
αIgM	+/+	38.0 ± 3.0	25.5 ± 2.6	16.2 ± 2.8
	+/–	30.1 ± 6.0	22.1 ± 5.3	9.1 ± 1.8
	–/–	28.2 ± 3.6	15.2 ± 3.0	5.8 ± 2.0
LPS	+/+	51.0 ± 6.6	51.6 ± 4.5	33.2 ± 4.1
	+/–	46.2 ± 6.7	48.9 ± 5.3	31.5 ± 2.9
	–/–	47.5 ± 6.9	42.8 ± 6.2	34.8 ± 4.0

stimulated through their B cell receptors; therefore *Spi-B*$^{–/–}$ B cells may be regulated by a mechanism similar to that in immature and tolerant B cells. It has been reported

that tolerant B cells display improper Ig signaling and are susceptible to Fas-induced apoptosis (Cooke *et al.*, 1994; Rathmell *et al.*, 1996). Fas-induced apoptosis appears to regulate tolerant B cell survival in the periphery (Rathmell *et al.*, 1995, 1996). As described by Rathmell *et al.*, this Fas-induced apoptosis can be blocked by strong Ig signaling. We have demonstrated that *Spi-B*$^{-/-}$ B cells up-regulate Fas surface expression upon anti-IgM cross-linking (data now shown). Since *Spi-B*$^{-/-}$ B cells suffer from a BCR-specific survival defect, Ig signaling may be insufficient to block Fas-mediated apoptosis.

The *Spi-B*$^{-/-}$ mice represent a highly useful model system for the study of B cell survival in response to BCR stimulation and the differences between T-independent and T-dependent responses.

Materials and methods

Targeting construct and generation of Spi-B$^{-/-}$ ES cells

The targeting construct was designed to delete the entire DNA-binding domain in exon 6 and part of the adjacent 5′ intron. mSpi-B was subcloned into the pPNT (Tybulewicz *et al.*, 1991) vector containing PGK::*neo* and PGK::*TK*. The *Not*I and *Xho*I sites of the 5′-homologous region (3.5 kb) were generated by PCR for subcloning purposes. The 3′-homologous region (5.1 kb) was subcloned at a *Bam*HI site. The targeting construct was linearized at the *Not*I site, electroporated into R1 ES cells, and cells were selected in 0.25 mg/ml G418 and 1 mM gancyclovir as described (Scott *et al.*, 1994). *Spi-B*$^{-/-}$ ES cells were generated with high G418 selection.

Northern blot analysis

Splenocytes were processed into single cell suspension and treated with ammonium chloride lysis buffer (0.017 M, pH 7.65 and 0.14 M NH_4Cl). RNAs were obtained from splenocytes using Trizol (GIBCO BRL, Gaithersburg, MD) and mRNAs were then made from the total RNAs following the Oligotex Poly(A)$^+$ RNA purification protocol (QIAGEN, Chatsworth, CA). Poly(A)$^+$ Northern blot analyses were done as previously described (Su *et al.*, 1996).

In vitro differentiation

Spi-B$^{+/+}$ and *Spi-B*$^{-/-}$ ES cells were adapted from mitomycin-C treated SNL feeder cells to 0.1% porcine skin gelatin (Sigma, St Louis, MO) coated tissue culture plates. ES cells (6×10^3 to 1.5×10^4) were placed in 35 mm bacterial Petri dishes with 1.5 ml of 0.9% methylcellulose media supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), kit ligand (25 ng/ml, Genetics Institute, Boston, MA), IL-1α (7.5×10^2 U/ml, Hoffman-LaRoche, Nutley, NJ), IL-3 (7 U/ml, Genetics Institute), 0.033% GM–CSF (Genetics Institute), erythropoietin (2 U/ml, R&D System, Minneapolis, MN) and α-monothioglycerol (4.5×10^{-4} M, Sigma, St Louis, MO). The ES cells differentiated into embryoid bodies (EBs), which were harvested on day 11 for cytospins and RNA preparation. Cytospun EBs were stained with May–Grunwald–Giemsa.

Cell counts and flow cytometry analysis

Single cell suspension was prepared from spleens and lysed with ammonium chloride buffer, and total cell numbers were obtained by trypan-blue exclusion in a hemacytometer. For flow cytometry analysis, each sample was stained with FITC-labeled monoclonal antibody (CD8 or IgM; PharMingen, San Diego, CA) and PE-conjugated monoclonal antibody (CD4 or B220; PharMingen) as reported (Su *et al.*, 1996). Labeled cells were analyzed by the LYSIS II program of a FACScan instrument (Becton and Dickinson, Gaithersburg, MD) and subsequently quantitated by WINmidi software. Ten thousand events were collected per dot plot.

Serum immunoglobulin isotyping and antigenic challenges

Mice were immunized with 10 μg of DNP–LPS or DNP–KLH by intraperitoneal injection. The secondary injection of DNP–KLH was introduced 30 days after the primary challenge. Donkey anti-mouse Ig (Jackson Immuno Research Laboratories, Inc., West Grove, PA) or DNP–BSA was used as the capturing reagent for basal serum Ig titer or antigen-specific serum Ig titer respectively. The isotype-specific secondary antibodies were from the Clonotyping System/AP (Southern Biotechnology Associates, Inc., Birmington, AL). The enzymatic reactions were developed with p-NPP Phosphatase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Each optical density value was converted to Ig concentration by using purified mouse anti-TNP, IgG_{2a} or κ standards (PharMingen).

Immunohistochemistry

Spleen sections of mice post-DNP–KLH injection were fixed in 4% paraformaldehyde and embedded in paraffin. Four μm sections of each sample were mounted onto superfrost plus slides, baked at 60°C for 1 h, cleared in xylene and hydrated through a descending alcohol series to distilled water. Slides were microwaved for antigen retrieval and allowed to cool for 15 min, followed by washes under running water. Endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide in methanol for 20 min. Tissue sections were then incubated with either biotinylated B220 (PharMingen) or the lectin PNA (Vector Laboratories, Burlingame, CA) overnight at 4°C. Normal mouse serum Ig (Ventana Medical Systems, Tucson, AZ) was used as negative control. The immunohistochemical staining was performed on a Ventana Gen system (Ventana Medical System) which utilizes streptavidin conjugated with horseradish peroxidase for detecting the immunocomplex and a diaminobenzidine as substrate for localization. The immunostained sections were counterstained with hematoxylin and dehydrated through an ascending alcohol series.

For TUNEL assay, the hydrated sections were prepared essentially as described by Gavrieli *et al.* (1992). The tissue sections were counterstained with nuclear fast red and dehydrated through an ascending alcohol series.

Cell purification and proliferation

B cells were purified from adhesion-depleted splenocytes via anti-Thy-1 (AT83A cocktail, a gift from Dr J.Bluestone)/Rabbit Complement (Pel-Freeze, Rogers, AK) killing. Lysed cells were removed using a Ficoll gradient. T cells were purified from lymph nodes using T-cell-enrichment-columns (R&D Systems, Minneapolis, MN). The purity of the B and T cells was >95% as assayed by flow cytometry. 2×10^6 cells/ml of B cells and 4×10^6 cells/ml of T cells were added in each assay. Ninety-six well plates were precoated with Anti-CD3 (PharMingen) for 2 h at 37°C. Anti-IgM antibody (50 μg/ml, affinity pure $F(ab')_2$ fragment, goat anti-mouse IgM, μ chain specific, Jackson Immuno Research Laboratories, Inc., West Grove, PA), LPS (50 μg/ml), ConA (2 μg/ml) and PMA (10 ng/ml) + ionomycin (0.5 μg/ml) were given as soluble stimulants. Cultures were pulsed with [^{3}H]thymidine at 1 mCi/ml (specific activity = 2 Ci/mmol; Amersham, Arlington Heights, IL) after 48 h, harvested and counted 16 h after thymidine treatment.

Flow cytometry-based analyses on activated B cells

B cells were purified and incubated with anti-IgM, LPS or media alone as described above. Activated B cells were harvested and stained with anti-CD69, anti-MHC class II, anti-CD19 and anti-CD44 (PharMingen) at 24, 48 and 72 h after stimulation for flow cytometry analysis following the standard staining protocol. CD69 FACS profile was displayed by gating on a lymphocytic population based on forward versus propidium iodide in the WINmidi software (10 000 events counted per plot).

For cell cycle analysis, stimulated cell samples were harvested and stored in PBS containing 70% EtOH for between 30 min at room temperature to a week at 4°C. Fixed samples were subsequently treated with RNase A and analyzed in the presence of propidium iodide staining solution as previously reported (Crissman and Steinkamp, 1973). Fifty thousand events were counted from the anti-IgM-stimulated cells. Cell cycle data were analyzed by WINCYCLE and graphed by WINmidi software programs. Clumped cells were excluded from the analysis and graph.

To compile the cell survival table, percentages of live cells were obtained from trypan blue, propidium iodine, 7-AAD or annexin V exclusion (R&D).

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