Developing B cells undergo a series of highly ordered maturation steps that result in the expression of a mature form of the BCR on the cell surface. Expression of a mature BCR first occurs at the immature B cell stage, and approximately half of these newly generated receptors have been shown to react with self-antigens (Grandien et al., 1994; Casellas et al., 2001; Wardemann et al., 2003). Normally, cells expressing a BCR that recognizes self-antigens are negatively selected and prevented from entering the mature peripheral B cell compartment (Pelanda et al., 1997; Halverson et al., 2004). In contrast, cells that express nonautoreactive BCRs enter the peripheral circulation and migrate to the spleen, where they undergo further differentiation (Pelanda et al., 1997; Halverson et al., 2004). Therefore, the differentiation step of immature B cells into transitional B cells is important for the generation of the primary naive B cell repertoire.

It is well documented that engagement of antigen by the BCR activates a signaling cascade that mediates antigen-specific responses. Studies conducted over the past decade have indicated that the BCR is also capable of signaling in the absence of antigen binding (Monroe, 2006). This ligand-independent, or tonic, BCR signal has been reported to be involved in regulating peripheral B cell survival as well as early B cell development. Specifically, gene ablation studies showed that deletion of the BCR leads to a dramatic loss of transitional and mature B cells in the spleen, and that this loss can be delayed by constitutive Bcl-2 expression (Lam et al., 1997). Subsequently, it was determined that the signaling capacity of the BCR, and not solely its expression, is critical for the maintenance of peripheral B cells (Meffre and Nussenzweig, 2002; Kraus et al., 2004). Specific to B cell development, it was reported that when tonic BCR signaling is interrupted through either the use of chemical inhibitors or inducible gene deletion, immature B cells undergo a developmental regression and express a gene profile similar to that of pro– and pre–B cells, and have reduced transcription of genes encoding mature B cell markers (Tze et al., 2005).

The specific signaling components of the tonic and antigen-mediated BCR pathways have yet to be fully elucidated, and whether these pathways are only quantitatively or also
qualitatively distinct is still under investigation. Ablation of BCR genes has been useful for recognizing the existence of tonic BCR signaling, but because this leads to cell death it has not allowed the analysis of the nature of tonic BCR signaling and its role in B cell development. To bypass this issue, we used a novel mouse strain that carries two differentially targeted alleles of the mb-1 gene, resulting in hypomorphic expression of the wild-type form of Ig-α. In these mice, reduced expression of Ig-α translates into comparably reduced expression of the BCR that manifests as reduced tonic BCR signaling. In this study, we used BCR-low mice to investigate whether tonic BCR signaling is required for the selection of nonautoreactive immature B cells from the bone marrow into the peripheral lymphoid system and for their differentiation into transitional and mature peripheral B cells.

Our study shows that nonautoreactive immature B cells with low surface BCR expression are capable of, but inefficient at, reaching the peripheral B cell compartment and differentiating into transitional and mature B cells. Furthermore, we show that reduced numbers of transitional and mature B cells in BCR-low mice is primarily caused by a defect in the differentiation capacity of immature B cells rather than reduced cell survival. Finally, we show that the differentiation of normal immature B cells requires the activation of extracellular signal-regulated kinase (Erk), and that the impaired differentiation of BCR-low immature B cells can be rescued by activating the Ras pathway, via an Erk-dependent mechanism.

RESULTS

Generation of nonautoreactive BCR-low mice

Ig-α associates with Ig-β and the H and L chains to form the BCR, and all protein subunits are absolutely required for surface receptor expression (Hombach et al., 1990). Therefore, low Ig-α levels can result in reduced BCR expression and, presumably, in low BCR signaling. We previously generated conditional mb-1 mice in which a cDNA cassette encoding wild-type exons II–V replaced the equivalent genomic exons (Pelanda et al., 2002a,b). Although the targeted allele expressed a wild-type form of Ig-α, the expression level was reduced relative to that of the genomic allele. The reason for this difference is presently unknown but likely involves the disruption of a transcription regulatory element within the targeted allele. Conditional mb-1 mice were bred to Ig-α-deficient (mb-1 null) mice (Pelanda et al., 2002a,b) to generate hemizygous animals named mb1-low (Fig. 1 A). To study the role and nature of tonic BCR signaling in the differentiation of nonautoreactive immature B cells, we bred mb1-low mice to 3-83Igi,H-2d (Pelanda et al., 1997; Braun et al., 2000; Halverson et al., 2004) and B1-8Hi/3-83ki (Pelanda et al., 1997) H and L chain–targeted mice to generate 3-83Igiflow and B1-8Hi/3-83ki-low mice, respectively. It has been well established that both the 3-83H/3-83k– and B1-8Hi/3-83k– expressing B cells are nonautoreactive on an H-2d genetic background (Nemazee and Bürki, 1989; Lang et al., 1996; Pelanda et al., 1997; Novobrantseva et al., 2005). Therefore, B cells from both 3-83Igiflow and B1-8Hi/3-83ki-low mice are nonautoreactive and hypomorphic for Ig-α. The experiments reported in this paper were performed on 3-83Igilow mice, and most were repeated on B1-8Hi/3-83ki-low animals to confirm that the results were independent of antibody specificity.

Total bone marrow cells from 3-83Igi and 3-83Igilow mice were cultured in IL-7 for 4 d to enrich for B cells, and total cell lysates from the cultured cells were analyzed by Western blotting to quantify Ig-α and actin (Fig. 1 B). Band intensities for Ig-α were normalized to those of actin, and the normalized Ig-α levels of 3-83Igi cells were set to 100%. These analyses found that Ig-α levels in 3-83Igi-low bone marrow B cells were ~12% (12.4 ± 6.4%; n = 3) of those in 3-83Igi cells. The hypomorphic expression of Ig-α in 3-83Igilow B cells resulted in comparably reduced expression of surface IgM (Fig. 1 C), which was ~18% (as calculated from the ratio of mean fluorescence intensities [MFIs] that follow) of the normal level on immature B cells on average, and was maintained at a similar level throughout B cell development (not depicted). The degree to which IgM expression was reduced was calculated by comparing the MFIs of surface IgM expression on 3-83Igi (179.4 ± 47.3; n = 5) and 3-83Igilow (32.9 ± 7.8; n = 5) bone marrow B220+IgM+IgD− immature B cells. In similar analyses, IgD expression was found to be ~40% of normal levels on 3-83Igilow transitional B cells (3-83Igi: 28.6 ± 3.7 [n = 3]; 3-83Igilow: 12.7 [n = 3]) and reached 70% of normal levels on mature B cells (3-83Igi: 114.6 ± 2.5 [n = 3]; 3-83Igilow: 83.5 ± 12.5 [n = 3]) in the spleen (unpublished data). IgD has been suggested to possess higher affinity for Ig-α/β than IgM (Schamel and Reth, 2000), potentially explaining the differential effect of hypomorphic Ig-α on their expression. Nevertheless, low Ig-α expression translated into low BCR expression at every B cell stage. It is important to note that despite the reduced level of surface BCR, the large majority of the peripheral B220+IgM+ B cells in 3-83Igilow (94.1 ± 3.2% 3-83Ig+ cells; n = 4) and B1-8Hi/3-83ki-low (95.3 ± 3.5% B1-8Hi3-83k+ cells; n = 6) mice retained the original specificity, indicating minimal receptor editing during B cell development (unpublished data).

BCR-low mice manifest reduced numbers of transitional and mature B cells

Initial characterization of 3-83Igilow mice revealed significantly reduced numbers of B cells in late stages of bone marrow development and in all B cell subsets in the spleen (Fig. 1, D–F). Although numbers of bone marrow immature B cells were comparable in 3-83Igi and 3-83Igilow mice (Fig. 1 E), numbers of transitional and mature B cells in both bone marrow and spleen were reduced two- to fourfold (Fig. 1, E and F). Similar differences in transitional and peripheral mature B cell numbers were observed in B1-8Hi/3-83ki-low mice when compared with B1-8Hi/3-83ki control animals (Fig. S1), as well as in mb1-low mice carrying a normal Ig repertoire, for the B cell populations analyzed (Fig. S2). These findings indicate that a threshold level of BCR expression is required for the differentiation of immature into transitional B cells, and potentially also of transitional into mature B cells,
independently of antibody specificity. Moreover, they also suggest that low BCR expression translates into suboptimal tonic BCR signaling and that an optimal level of signaling is required for B cell development, as previously suggested (Kouskoff et al., 2000).

To uncover the full effect of low BCR expression on B cell development, mixed bone marrow chimeras were set up to compare the ability of 3-83Igi-low and control 3-83Igi B cells to develop alongside wild-type (CB17) B cells (Fig. 2 A). Cells from each donor were distinguished using the allootypic markers IgM\(^a\) (on 3-83Igi and 3-83Igi-low cells) and IgM\(^b\) (on CB17 cells). In agreement with our previous study (Halverson et al., 2004), 3-83Igi B cells developed normally in the presence of wild-type cells, maintaining a similar frequency from the immature to the mature B cell stages (Fig. 2, B and C). In contrast, the frequency of 3-83Igi-low B cells decreased and that of CB17 B cells reciprocally increased as the cells progressed from the immature to the transitional and mature B cell stages, indicating an impaired ability of 3-83Igi-low B cells to differentiate from the immature to the transitional and from the transitional to the mature B cell stages (Fig. 2, B and D). This result was supported by a reduction in absolute B cell numbers of 3-83Igi-low cells relative to 3-83Igi and CB17 cells in bone marrow chimeras (Table S1).

**BCR-low mature B cells have decreased survival in vivo but not in vitro**

B cell activating factor (BAFF) is a cytokine required for the differentiation of transitional into mature B cells and for the survival of both B cell types (Schneider et al., 1999; Thompson et al., 2000; Harless et al., 2001). In addition, a recent study reported that BAFF-R signaling is dependent on functional tonic BCR signaling (Stadanlick et al., 2008). Thus, it was conceivable that a reduced ability to respond to BAFF could explain...
the diminished numbers of mature B cells in 3-83Igi-low mice. However, when we compared the survival of 3-83Igi-low and 3-83Igi spleen B cells in culture with or without BAFF, we found that the number of viable cells was similar in 3-83Igi and 3-83Igi-low cultures regardless of the presence of BAFF (Fig. 3 A). These results indicate that 3-83Igi-low mature B cells had normal intrinsic and BAFF-mediated survival in vitro.

In addition to BAFF, BCR expression and signaling have been shown to be required for B cell survival in vivo (Lam et al., 1997; Meffre and Nussenzweig, 2002; Kraus et al., 2004). To test cell survival in vivo, we used a cell-adoptive transfer approach to compare the lifespan of 3-83Igi and 3-83Igi-low mature B cells to that of B1-8Hi/3-83ki B cells, which express normal levels of an innocuous BCR and served as a reference population (Fig. 3 B). Spleen B cells from either 3-83Igi or 3-83Igi-low mice were mixed in equal numbers with B1-8Hi/3-83ki B cells and transferred into B cell-deficient mb1−/− mice (Pelanda et al., 2002a). The mixed populations were analyzed by flow cytometry to measure the frequency of B1-8Hi/3-83ki cells in the total B cell population on day 0 before adoptive transfer, and on days 21 and 41 after transfer. Any change in the frequency of B1-8Hi/3-83ki B cells during this time would indicate a reciprocal change in the frequency of 3-83Ig+ B cells. In 3-83Igi:B1-8Hi/3-83ki adoptively transferred mice, 3-83Igi B cells were recovered on day 41 at a frequency that was similar to that of day 0 just before transfer, indicating that 3-83Igi and B1-8Hi/3-83ki mature B cells had a similar lifespan in vivo (Fig. 3 C). In contrast, the frequency of 3-83Igi-low B cells at days 21 and 41 was slightly (1.7-fold at day 41), but statistically significantly, reduced relative to the initial input. These results indicate a reduced lifespan of the 3-83Igi-low mature B cell population relative to the B1-8Hi/3-83ki B cell population in vivo, and may explain why the frequency of 3-83Igi-low mature B cells was further reduced relative to that of transitional B cells in mixed bone marrow chimeras (Fig. 2 D).

Constitutive Bcl-2 expression fails to rescue the differentiation of BCR-low immature into transitional and mature B cells

The finding that mature 3-83Igi-low B cells had a reduced lifespan in vivo suggested that a similar defect in the survival of immature B cells might be the cause of reduced transitional B cell numbers. In addition, genetic ablation of the BCR has been shown to cause death of mature as well as

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**Figure 2.** BCR-low B cells have impaired development in competition with normal B cells. (A) Schematic for the generation of mixed bone marrow chimeras. (B) Cells from mixed bone marrow chimeras described in A were analyzed by flow cytometry for the expression of IgM<sup>a</sup> and IgM<sup>b</sup>. Numbers represent the percentage of IgM<sup>a</sup> (top, 3-83Igi; bottom, 3-83Igi-low) and IgM<sup>b</sup> (CB17) cells within the total B220<sup>+</sup> cell population in the bone marrow (left) and spleen (right). (C and D) Mean frequencies (±SD; n = 6–10 mice from two separate experiments) of 3-83Igi, CB17, and 3-83Igi-low donor cells in mixed 3-83Igi:CB17 (C) and 3-83Igi-low:CB17 (D) bone marrow chimeras. The following surface markers were used to discriminate B cell subsets: immature (bone marrow), IgM<sup>a</sup>IgD<sup>+</sup>; transitional (bone marrow), IgM<sup>a</sup>IgD<sup>lo</sup>; transitional (spleen), B220<sup>+</sup>CD24<sup>hi</sup>; and mature (spleen), B220<sup>+</sup>CD24<sup>lo</sup>. N.S., nonsignificant p-values of 0.41–0.43.
immature B cells in vivo (Lam et al., 1997; Meffre and Nussenzweig, 2002). In light of these facts, we performed a flow cytometric characterization of Bcl-2 expression in B cells from 3-83Igi and 3-83Igi-low mice because Bcl-2 has been well characterized as a potent prosurvival molecule and is known to function in this capacity in developing B cells (Matsuzaki et al., 1997; Lu et al., 1999). These analyses indicated significantly reduced expression of Bcl-2 at all stages of 3-83Igi-low B cell development. Immature and transitional 3-83Igi-low B cells were particularly affected, showing Bcl-2 staining levels close to isotype control (Fig. 4A and Fig. S3).

To test whether differences in Bcl-2 expression and, potentially, in cell survival accounted for reduced transitional and mature B cell numbers in 3-83Igi-low mice, mixed bone marrow chimeras were set up to assess the ability of 3-83Igi-low B cells to differentiate alongside wild-type (CB17) B cells when Bcl-2 was constitutively expressed (Fig. 4B). Bone marrow cells from 3-83Igi-low (IgM⁺) and CB17 (IgM⁺) donors were transduced with bcl-2 to prevent a survival advantage mediated by Bcl-2 constitutive expression. The transduction efficiency at the time of cell transfer was ~40% for CB17 and 30% for 3-83Igi-low cells (unpublished data), and an equal number of cells from each donor were transferred into each recipient. Transduced cells were not purified before transfer, thereby providing an internal control of nontransduced cells for comparison. Thus, the recipient mice displayed four distinct B cell populations: CB17 and 3-83Igi-low, which were either transduced or not. As depicted, Bcl-2 levels in transduced 3-83Igi-low B cells were equivalent to those of transduced CB17 cells (Fig. 4C) and higher than those of intact 3-83Igi-low cells (Fig. 4A). Moreover, retrovirus-encoded Bcl-2 was functional as determined by greater recovery of transduced CB17 over nontransduced bone marrow and spleen cells (Fig. 4F and Table S1), and by prolonged lifespan of these transduced cells in in vitro cell culture (not depicted).

Surprisingly, constitutive Bcl-2 expression did not restore the ability of 3-83Igi-low immature B cells to differentiate in vivo. As depicted, the frequency (Fig. 4, D and E) of 3-83Igi-low B cells relative to that of wild-type cells decreased equally from immature to mature B cell stages regardless of Bcl-2 overexpression. Furthermore, when analyzing absolute cell numbers, an expansion of wild-type (CB17) B cells was observed in the transitional and mature B cell populations relative to the immature B cell population, and this expansion was amplified by Bcl-2 constitutive expression (Fig. 4F and Table S1). In contrast, the transitional and mature 3-83Igi-low B cell populations did not expand but displayed a contraction relative to their immediate precursors, a contraction that was not overcome by Bcl-2 constitutive expression (Fig. 4F and Table S1). Therefore, these data indicate that reduced Bcl-2 expression and decreased B cell survival are not the primary causes for the impaired development of 3-83Igi-low B cells.

**Low BCR expression inhibits the differentiation of immature B cells in an in vitro system**

The observation that 3-83Igi-low B cells were greatly reduced in the peripheral lymphoid tissue, even when overexpressing the prosurvival protein Bcl-2, suggested that B cells with low BCR expression had a specific defect in differentiation. This idea agrees with the previous finding that BCR deletion causes the “back-differentiation” of immature B cells into cells with a pre-B cell–like phenotype (Tze et al., 2005), implying that tonic BCR signaling is required to maintain the differentiation state of immature B cells.

To further study the role and nature of tonic BCR signals in the differentiation of immature B cells, we developed an in vitro differentiation system that allows us to follow the
expression of cell-surface markers associated with B cell maturation, as well as to genetically and pharmacologically manipulate this process. In this system, bone marrow cells from 3-83Igi and 3-83Igi-low mice are cultured in the presence of IL-7 for 3–4 d to enrich for immature B cells. In accordance with published studies using 3-83Ig targeted and transgenic mice (Melamed et al., 1997; Braun et al., 2000), IL-7 cultures of 3-83Igi (and 3-83Igi-low) bone marrow cells generated a population of >90% B220+IgM+ cells (Fig. S4 and not depicted) that were CD43−/low, CD2+, CD21−/low, CD23−, and mostly IgD− (Fig. 5, A and B; and Fig. S4). Based on this phenotype, these cultures represent a population of immature B cells and not of pro- or pre–B cells. After the IL-7 culture, cells are replated in medium containing BAFF to enhance B cell differentiation and survival (Claudio et al., 2002). On subsequent days of BAFF culture, cells are evaluated by flow cytometry for the expression of B cell maturation markers. As shown in Fig. 5 (A–C), the in vitro differentiation system recapitulated the in vivo results, with many 3-83Igi immature B cells differentiating to a cell stage that expressed CD21, CD23, and IgD by day 2 of BAFF culture, whereas 3-83Igi-low cells remained, for the most part, negative for these markers. Similar

Figure 4. Constitutive expression of Bcl-2 fails to rescue the development of 3-83Igi-low B cells. (A) Flow cytometric analysis of endogenous Bcl-2 levels in bone marrow and spleen B cells from 3-83Igi and 3-83Igi-low mice. B cell subsets were defined as in Fig. 2 (C and D). Data represent the geometric MFIs ± SD from six mice from six separate analyses. The dotted line just above the x-axis represents the average geometric MFI of isotype control staining. (B) Schematic for the generation of bcl-2 retrogenic mice. (C) Bcl-2 levels in CB17 and 3-83Igi-low transduced (Thy1.1+) bone marrow and spleen B cells from bcl-2 retrogenic mice generated as described in B. Data represent the geometric MFIs ± SD of five mice from one experiment. (D and E) Frequency of transduced (D) and nontransduced (E) 3-83Igi-low and CB17 donor cells throughout B cell development in bcl-2 retrogenic mice described in B. (F) Absolute numbers of B220+ transduced and nontransduced donor-derived B cells within the immature, transitional spleen, and mature spleen B cell populations in bcl-2 retrogenic mice described in B–E. B cell subsets were defined as in Fig. 2 (C and D). Data represent the arithmetic means ± SD of five mice from one experiment. Similar results were obtained in an additional independent experiment with five retrogenic mice.
delayed kinetics of differentiation, we introduced Bcl-2 to maintain longer cell survival. We found, however, that Bcl-2 constitutive expression maintained the survival of undifferentiated immature B cells after IL-7 withdrawal. This caused a reduction in the frequency of differentiated cells in 3-83Igi cultures (Fig. 5 D). Nevertheless, CD21 and CD23 up-regulation could still be used to follow cell differentiation.

Figure 5. Low BCR expression inhibits differentiation of immature B cells in vitro. (A) Representative kinetics of immature B cell differentiation in vitro. Bone marrow cells from 3-83Igi and 3-83Igi-low mice were cultured in the presence of IL-7 for 4 d. After IL-7 culture (day 0), cells were recultured in the presence of BAFF and B220+ cells were analyzed by flow cytometry for expression of CD21, CD23, and IgD at the indicated times. (B) Representative flow cytometric analysis of CD21 and CD23 expression in 3-83Igi (top) and 3-83Igi-low (bottom) immature B cells at the indicated time of BAFF culture as described in A. Numbers indicate frequencies of live B220+ B cells in quadrant gates. (C) Mean frequency (±SD; n = 3 mice from three individual experiments) of CD23+CD21+ B cells at day 2 of BAFF culture as described in B. (D) Representative analysis of CD21 and CD23 expression of bcl-2 transduced 3-83Igi (top) and 3-83Igi-low (bottom) immature B cells at days 0 and 6 of BAFF culture as described in A. Numbers indicate frequencies of live transduced (Thy1.1+) B220+ B cells in quadrant gates. (E) Mean frequency (±SD; n = 2 pooled mice in each of three individual experiments) of CD21+ (left) and CD23+CD21+ (right) bcl-2 transduced 3-83Igi and 3-83Igi-low B cells analyzed as in D at day 6 of BAFF culture.
In agreement with the bone marrow chimera studies (Fig. 4), Bcl-2 overexpression failed to restore normal differentiation of 3-83Ig−low immature B cells cultured for up to 6 d, as indicated by a lower frequency of CD21+ and CD21+CD23+ cells relative to bcl-2–transduced 3-83Ig+ cultures (Fig. 5, D and E). However, the in vitro defect was not absolute and a small fraction of 3-83Ig−low immature B cells (20%) displayed markers of differentiation, as shown by up-regulation of CD21 (Fig. 5, D and E). These analyses indicate that optimal BCR expression and signaling are needed to promote development beyond the immature B cell stage in vitro as well as in vivo. However, some differentiation can occur in cells expressing suboptimal levels of BCR when cell survival is artificially prolonged, at least in vitro.

Constitutive activation of Ras restores the differentiation of 3-83Ig−low immature B cells in vitro and in vivo

It is well known that Erk is a major downstream effector of the BCR signaling pathway. To determine whether Erk mediates tonic BCR signals that are involved in immature B cell differentiation, we compared the levels of phosphorylated Erk (pErk) in 3-83Ig+ and 3-83Ig−low B cells by flow cytometric analyses. In untreated B cells, pErk was only weakly and inconsistently detected (unpublished data). Therefore, we evaluated pErk in B cells treated with sodium pervanadate to amplify the low BCR signal of resting cells (Wienands et al., 1996). The levels of pErk were higher, on average, in mature than in transitional and immature B cells of both strains. Importantly, however, 3-83Ig−low B cells had decreased expression of pErk relative to 3-83Ig+ at all stages of development, as indicated in repeated paired analyses of the two strains, and this difference was more significant at the immature and transitional than the mature cell stages (Fig. 6 A and Fig. S6). Erk has been previously shown to be required for pro–to pre–B cell differentiation and for the response of pre–B cells to IL-7, suggesting its involvement in pre-BCR signaling (Fleming and Paige, 2001; Milne et al., 2008; Yasuda et al., 2008). These previous findings and our results led us to hypothesize that Erk plays a critical role in mediating the differentiation of immature B cells downstream of tonic BCR signals.

As Erk is directly activated by the mitogen-activated protein kinase (MAPK) kinase (MEK), we used the specific MEK inhibitor U0126 (Favata et al., 1998; Urosevic et al., 2009) to prevent Erk activation in the in vitro B cell differentiation assay. As shown in Fig. 6 (B and C), a significantly reduced frequency of CD21+CD23+ 3-83Ig− B cells was observed in cultures in which MEK was inhibited, whereas cell differentiation was unaffected by the p38 MAPK inhibitor SB203580. These data, therefore, suggest that the activation of Erk, and not that of another MAPK, was important for immature B cell differentiation.

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Figure 6. Inhibition of Erk activity impairs the in vitro differentiation of normal immature B cells. (A) Bone marrow (immature) and spleen (transitional and mature) B cells from 3-83Ig+ and 3-83Ig−low mice were treated with pervanadate and analyzed by intracellular flow cytometry for expression of pErk. B cell subsets were defined as in Fig. 2 (C and D). Data represent the geometric MFIs from four mice and four individual experiments. Matching filled and open symbols represent mice that were analyzed at the same time (paired samples). The short black bars indicate average MFIs, and the dashed line just above the x-axis represents the average geometric MFI of isotype control staining. (B) Representative flow cytometric analysis of CD21 and CD23 expression in 3-83Ig+ immature B cells cultured as described in Fig. 5 A, with the exception that cells were treated with 10 μM U0126 (MEK inhibitor) in 0.04% DMSO, 10 μM SB203580 (p38 inhibitor) in 0.3% DMSO, or respective amounts of DMSO only during BAFF culture. Cells were analyzed at days 0 and 2 of BAFF culture. Numbers indicate frequencies of live B220+ B cells in quadrant gates. (C) Mean frequency (±SD; n = 3 mice from three individual experiments) of CD23+CD21+ 3-83Ig− B cells at day 2 of BAFF culture as described in B.
Our analyses clearly show the presence of a large fraction of CD23^+CD21^+ (and IgD^+; not depicted) 3-83igi-low cells expressing constitutively active N-RasD12 after IL-7 withdrawal, whereas CD23^+CD21^+ cells were not observed in control cultures transduced with gfp only (Fig. 7, A and B). Similar findings were obtained after N-RasD12 transduction of B1-8Hi/3-83ki-low immature B cells (unpublished data). Moreover, N-RasD12 transduction further improved the differentiation of BCR-normal 3-83Igi B cells, generating…

To further test a role for Erk in immature B cell differentiation, we promoted Erk activity by using a constitutively active form of human N-Ras that carries a G to D single amino acid substitution at position 12 (N-RasD12; Bos, 1989; Scheffzek et al., 1997). Ras activation leads to the activation of MEK via Raf and, therefore, to the activation of Erk (Kolch, 2000). Immature B cells from 3-83Igi-low mice were transduced in vitro with retroviruses encoding either N-RasD12 or GFP-only control and assayed for in vitro B cell differentiation. To further test a role for Erk in immature B cell differentiation, we promoted Erk activity by using a constitutively active form of human N-Ras that carries a G to D single amino acid substitution at position 12 (N-RasD12; Bos, 1989; Scheffzek et al., 1997). Ras activation leads to the activation of MEK via Raf and, therefore, to the activation of Erk (Kolch, 2000). Immature B cells from 3-83Igi-low mice were transduced in vitro with retroviruses encoding either N-RasD12 or GFP-only control and assayed for in vitro B cell differentiation.
more CD21*CD23+ B cells after 2 d of BAFF culture (unpublished data). These results demonstrate that active N-Ras aids the differentiation of immature into transitional B cells and complements the differentiation defect of BCR-low immature B cells in vitro. To determine if N-RasD12 restored the differentiation of 3-83Igi-low and B1–8Hi/3-83ki-low B cells by specifically activating the Erk pathway, we used the MEK inhibitor U0126 to prevent Erk activation in transduced cells. We found that the differentiation of BCR-low immature into transitional B cells mediated by constitutively active N-Ras in vitro was inhibited by U0126 but not by the p38 inhibitor, strongly suggesting that Ras promoted immature B cell differentiation via Erk in vitro (Fig. 7, A and B; and not depicted).

To examine the ability of Ras to promote B cell development in vivo, we generated retrogenic mice by transducing 3-83Igi-low bone marrow cells with either gfp control or N-rasD12–encoding retroviruses. As done with bcl-2 retrogenic mice, we chose to assess the differentiation capacity of 3-83Igi-low B cells along that of wild-type CB17 B cells (Fig. 7 C). Chimeric mice were analyzed at 3 wk after transfer to limit tumor development caused by the expression of the constitutively active Ras oncogene (Braun et al., 2004; Chan et al., 2004). At this time, wild-type (CB17) B cells had progressed to the transitional T1 (CD24hiCD21–/lowCD23–/low) and T2 (CD24hiCD21hiCD23hi) stages, whereas fully mature CD24low B cells had not yet developed (Fig. 7 D). Because of the competitive advantage of N-rasD12–transduced cells, only a small frequency of CB17 B cells was detected in N-rasD12 retrogenic mice (unpublished data). Nevertheless, the majority of the CB17 spleen B cell population was composed of T2 cells in both retrogenic mice groups (Fig. 7, D and E; and not depicted), whereas B cells of the control gfp transduced 3-83Igi-low population were mostly at the T1 cell stage of maturation (Fig. 7, D and E). In contrast to control 3-83Igi-low cells, the population of N-rasD12–transduced 3-83Igi-low B cells was composed mainly of T2 cells in a similar proportion to that of the wild-type B cell population (Fig. 7, D and E). As with CB17 cells, only small numbers of nontransduced (GFP) 3-83Igi-low B cells were detected in N-rasD12 retrogenic mice because of the competitive advantage mediated by this oncogene in transduced cells. However, when detected, nontransduced (GFP) 3-83Igi-low B cells were mostly found at the T1 stage (unpublished data), indicating that the differentiation mediated by N-RasD12 in transduced cells is a cell-autonomous process. Therefore, in accordance with the in vitro studies, constitutive expression of active N-Ras restored the ability of 3-83Igi-low B cells to develop to the T2 cell stage in vivo. In fact, expression of active N-Ras permitted 3-83Igi-low B cells to differentiate comparably to wild-type B cells within the same individual mouse.

DISCUSSION

In this study, we provide evidence that tonic BCR signaling propagates via the Erk pathway to specifically mediate the development of immature B cells into transitional and mature B cells. Previous studies have shown that BCR deletion and, therefore, the total ablation of tonic BCR signals results in the back-differentiation of immature B cells into pre-B-like cells, indicating that the maintenance of the immature B cell stage requires active tonic BCR signaling (Tze et al., 2005). Our study extends those findings by showing that low BCR expression leads to suboptimal tonic BCR signals resulting in impaired differentiation of nonautoactive immature B cells into transitional and mature B cells. In our studies, we controlled the specificity of B cells through the use of targeted H and L transgenes (knockin). Low BCR expression had similar effects on B cell development whether the cells expressed the nonautoactive 3-83H3-83k or the B1–8Hi/3-83k antibody chain combinations, indicating that the phenotype was independent of the B cell specificity. Moreover, similar defects in B cell generation were observed in BCR-low mice with a normal polyclonal B cell repertoire. It is conceivable that suboptimal tonic BCR signals impair allelic exclusion and, consequently, activate receptor editing. Under this hypothesis, BCR-low immature B cells would be delayed in differentiation relative to B cells expressing normal BCR levels (Casellas et al., 2001). However, our analyses discount this possibility, as 94–95% of peripheral IgM+ B cells in both 3-83Igi-low and B1–8Hi/3-83ki-low mice expressed the original specificity (unpublished data). In our experiments, we used mb-1 hemizygous mice in which immature B cells expressed 18% of the normal IgM levels. In additional experiments, we analyzed 3-83Igi mice carrying two conditional mb-1 alleles (3-83Igi-int mice) that displayed a less severe hypomorphism characterized by IgM levels that were 50–70% of normal, and follicular B cell numbers that were intermediate between those of 3-83Igi and 3-83Igi-low mice (Fig. 5, A and B). We found, however, that immature B cells from 3-83Igi-int mice were still severely affected in differentiation in vitro, although to a lower extent than in 3-83Igi-low mice (Fig. 7 C). It is worth noting that IgM expression is widely distributed (over a 2-log range) in immature B cells and that ~40% of 3-83Igi-low immature B cells displayed IgM levels within the low end of expression of 3-83Igi immature B cells. This overlap in IgM expression might explain why a small fraction of BCR-low immature B cells was capable of further differentiation but also supports the idea that the IgMlow fraction of BCR-normal immature B cells may be impaired and/or delayed in differentiation, as previously proposed (Melamed et al., 1998). Overall, these findings suggest that in immature B cells, optimal tonic BCR signals depend on proper levels of IgM expression, and indicate that a threshold level of BCR expression is necessary to trigger a tonic BCR signal able to initiate the differentiation of immature B cells into transitional B cells.

Tonic BCR signals have been previously shown to be important for the maintenance of immature and mature B cell survival in vivo (Lam et al., 1997; Meffre and Nussenzweig, 2002; Kraus et al., 2004). However, enforced cell survival by increased Bcl-2 expression did not ameliorate the differentiation of 3-83Igi-low B cells either in vitro or in vivo. Although we cannot exclude that constitutive expression of a different
prosurvival molecule may restore the differentiation of BCR-
low B cells, our data argue against this possibility. In fact, al-
though both the bcl-2 and N-rasD12 genes promoted cell
survival as indicated by a longer lifespan in vitro (unpublished
cell data), bcl-2–transduced B cells remained mostly at the CD21–
CD23+ immature stage. In contrast, N-rasD12–transduced B
cells progressed to the transitional cell stage characterized by
CD21 and CD23 expression. Therefore, although cell sur-

vival is a prerequisite for cell differentiation, reduced cell sur-
vival was not the primary cause of the impaired differentiation
of BCR-low immature B cells. Our data strongly suggest that
tonic BCR signals propagated via Erk, likely through Ras,
are specifically required for the export of immature B cells
from the bone marrow and their differentiation into transi-
tional B cells.

In addition to a defect during bone marrow B cell devel-

opment, BCR-low mice were unable to accumulate normal
numbers of mature B cells in the spleen. This may be caused
by the fact that BCR-low mature B cells had a slightly re-
duced lifespan in vivo relative to BCR-normal B cells. Thus,
our findings agree with previous reports indicating that tonic
BCR signals contribute to the survival of mature B cells (Lam
et al., 1997; Meffre and Nussenzweig, 2002; Kraus et al.,
2004). In addition to this previously recognized function, our
data suggest that tonic BCR signals are also required for the
differentiation of transitional B cells into mature B cells. Evi-
dence for this novel role comes from the analysis of B cell
development in bcl-2 retrogene mice. Specifically, 3–83Igi-
low transitional B cells had impaired differentiation into ma-
ture B cells, even when provided with the prosurvival gene
bcl-2, as demonstrated by a decrease in the frequency and a
lack of expansion in numbers of B cells from the transitional
to mature B cell populations. These results, therefore, indicate
that in peripheral B cell populations, tonic BCR signals are
required for both the differentiation of transitional into ma-
ture B cells and the maintenance of the mature B cell pool.

The cytokine BAFF has been shown to contribute to the
development and survival of transitional T2 and mature B cells
(Gross et al., 2001; Sasaki et al., 2004). Both ligand-dependent
and -independent BCR signals have been recently reported
to generate the NF-κB p100 subunit that is required for func-
tional BAFF-R signaling in response to BAFF (Stadanlick
et al., 2008). Consequently, it is conceivable that BCR-low
immature B cells are impaired in differentiation because they
are unable to generate p100 and respond to BAFF. This sce-
nario, however, is unlikely for the following two reasons.
First, normal immature B cells differentiate to T1 B cells re-
gardless of BAFF or BAFF-R signaling (Gross et al., 2001;
Sasaki et al., 2004), whereas we show that BCR-low imma-
ture B cells did not efficiently progress to the T1 stage. Sec-
ond, 3–83Igi-low mature B cells had a normal response to
BAFF in vitro, again suggesting that the reduced expression of
BCR in these cells did not impair optimal p100 production.
Nevertheless, this issue requires further clarification.

The nature of tonic BCR signaling and how it differs
from that mediated by ligand is still unclear. We found that
immature and transitional B cells expressing suboptimal levels
of BCR had reduced amounts of pErk, suggesting that tonic
BCR signals are proportional to Erk phosphorylation. The pErk
analysis was performed on cells treated with pervanadate,
which is known to inhibit tyrosine phosphatases, thereby
amplifying the effect of kinases. Thus, our data suggest that
BCR-low–developing B cells have reduced expression and/or
activity of kinases upstream of Erk, which has not been pres-
tently determined. Recent studies have shown that the com-
bined deletion of Erk1 and Erk2 results in a complete block
in the differentiation of pro- to pre-B cells, and indicated
that the Erk pathway propagates pre-BCR signals required
for pre-B cell development (Yasuda et al., 2008). In our sys-
tem, we used Ig-targeted mice with prerearranged H and L
chain genes that allowed the progenitor B cells to advance to
the immature B cell stage without the requirement for pre-
BCR signaling and Ig gene recombination. Thus, this system
allowed us to look at the role of Erk in the progression of im-

mature into transitional and mature B cells. Our data strongly
suggest that activation of Erk is required for this differentia-
tion step. Erk requirement is indicated by the following find-
ings. Only a few 3–83Igi-low B cells reach the mature B cell
stage, and these cells display high levels of pErk that are simi-
lar to those of mature 3–83Igi B cells, suggesting a role for
Erk in the positive selection process of immature B cells. Ad-
ditionally, reduced differentiation of 3–83Igi normal immu-

nate B cells was observed when Erk activation was prevented
after MEK inhibition by U0126. Under similar conditions, a
MAPK p38 inhibitor did not alter the differentiation of nor-
mal immature B cells, indicating that not all MAPKs are
equivalent in mediating immature B cell differentiation. Fur-
thermore, differentiation of BCR-low immature B cells was
restored by expression of a constitutively active form of
N-Ras, a kinase that leads to the activation of Erk. Impor-
tantly, the differentiating function of N-Ras appeared to be
mediated via Erk, as it was inhibited by U0126 in vitro. Al-
though the inhibitor U0126 has been demonstrated to be
particularly specific to MEK, it cannot be excluded that this
inhibitor may target another molecule involved in B cell dif-
ferentiation. Similarly, Ras is known to activate other down-
stream molecular pathways, in addition to Erk, that also may
be involved in B cell differentiation. However, the use of these
complementary approaches strengthens our conclusion that
active Erk mediates the differentiation of immature into tran-
sitional B cells via a Ras–dependent pathway. An alternative
but less likely explanation is that N-RasD12 activates a differ-
ent downstream mediator of cell differentiation that happens
to be an additional target of U0126. The inactivation of Ras
has been previously shown to inhibit B cell development at
the pro- to pre-B cell transition (Iritani et al., 1997), the
same stage as shown for Erk1/2 deletion (Yasuda et al., 2008).
Furthermore, a constitutively active H-RasV12 was shown
to cause the progression of Rag1-deficient pro-B cells to cells
that, despite the absence of Ig expression, displayed the mature
markers CD21 and CD23 (Shaw et al., 1999). Overall, these
reports suggest that active Ras can propagate differentiation
signals in B cells, and also support our argument that both Ras and Erk activities are necessary during B cell development. It is relevant to note that Ras and Erk activities have also been implicated in thymocyte development. A recent study has elegantly shown that positive selection of thymocytes involves a specific compartmentalization of Ras/MAPK signaling at the Golgi instead of the plasma membrane (Daniels et al., 2006). Thus, in the future it will also be important to define whether a similar compartmentalization of these signaling molecules occurs during positive and negative selection of immature B cells.

We were unable to find mature, CD24low B cells in N-rasD12 retrogenic mice at 3 wk after the inception of chimerism. Unfortunately, the rapid tumorigenesis and death mediated by N-rasD12 prevented us from analyzing most mice at later time points. Nevertheless, we believe that the absence of mature B cells was caused by the short-term hematopoietic reconstitution and does not indicate that constitutively active Ras cannot mediate the development of mature B cells. This view is supported by the fact that even CB17 intact B cells did not reach the CD24low mature B cell stage in the same mice. Moreover, two N-rasD12 retrogenic mice that survived for 4 wk had 3-83Igi-low B cells at the mature, CD24low stage (unpublished data), agreeing with the idea that active Ras mediates the differentiation of immature B cells all the way to the mature cell stage of development. Constitutively active forms of Ras are known oncogenes that prolong the survival of transformed cells. In line with this knowledge, we observed that B cells expressing N-rasD12 survived longer in vitro (unpublished data). Nevertheless, N-RasD12 had a peculiar capacity to push BCR-low immature B cells to differentiate. This role appeared to be independent of the prosurvival function of Ras, as suggested by the fact that BCR-low immature B cell differentiation was not promoted by increased cell survival after constitutive expression of Bcl-2. Along this line, the tumors that developed in N-rasD12 retrogenic mice appeared to be of myeloid and not lymphoid origin (unpublished data), supporting the idea that Ras promotes differentiation, and not merely survival and proliferation, of B cells. Thus, our observations, together with those previously published, provide compelling evidence to support a model in which sufficient BCR levels trigger tonic BCR signals that activate Erk in a Ras-dependent manner to promote differentiation of nonautoreactive immature B cells into transitional and mature B cells.

In both mice and humans, the bone marrow immature B cell stage is an important checkpoint at which most autoreactive immature B cells are eliminated, whereas nonautoreactive B cells migrate to the spleen to generate the naive B cell repertoire (Grandid et al., 1994; Wardemann et al., 2003). Our study suggests that this checkpoint is a positive selection process regulated by proper activation of Ras and Erk. If so, modifications of the Ras and Erk signaling pathways would be expected to affect the peripheral B cell pool. In one scenario, altered Ras/Erk activation may lead to decreased peripheral B cell generation and consequent immunodeficiency. Conversely, it may lead to positive selection of immature B cells in the absence of proper tonic BCR signaling, a process that could involve autoreactive immature B cells and lead to the development of autoantibodies.

**MATERIALS AND METHODS**

**Mice.** The 3-83Ig (Igk +/−/−Igh 6/14H 2/2), Braun et al., 2000; Halverson et al., 2004), B1-8Hi/3-83ki (Igk +/−/−Igh 6/14H 2/2; Pelanda et al., 1997), mb-I null (mb-I−/−; Pelanda et al., 2002a), and mb-I conditional (mb-I−/−, mb-Icre/mb-Icre; Pelanda et al., 2002b) mice have been previously described. The mb-I−/− (mb-I−/−NKO-IgG2a), 3-83Ig−/− (Igk +/−/−Igh 6/14H 2/2sub-1−/−Tas1Cre/vo), 3-83Ig−/− (Igk +/−/−Igh 6/14H 2/2sub-1−/−Tas1Cre/vo), B1-8Hi/3-83ki-low (Igk +/−/−Igh 6/14H 2/2sub-1−/−Tas1Cre/vo), and 3-83Ig−/−/− (Igk +/−/−Igh 6/14H 2/2sub-1−/−Tas1Cre/vo) mice were generated for this study. CB17 mice were initially purchased from the Jackson Laboratory. All mice were on a BALB/c genetic background and were bred and maintained in specific pathogen-free rooms at the Biological Resource Center at National Jewish Health (NJH). All animal experiments were approved by the NJH Institutional Animal Care and Use Committee.

**Retroviral constructs and production of retroviral particles.** The pMSCV-Flag-Bcl2-IRES-Thy1.1 (a gift of A. Desbien and P. Marrack, NJH; Dubernet et al., 2009), pMSCV-IRES-GFP (Van Parps et al., 1999), and pMSCV-GFP-IRES-hN-RasG12D (a gift from J. DeGregori, University of Colorado Denver, Denver, CO) retroviral vectors encode replication-deficient viruses. The pCL-Eco vector is an ecotropic helper vector (Naviaux et al., 1996). Retroviruses were produced in Phoenix cells (Swift et al., 2001) using Lipofectamine 2000 Transfection Reagent (Invitrogen) optimized according to the manufacturer’s instructions. At 48 and 72 h after transfection, viral supernatants were collected and filtered through 0.45-µm filters, flash frozen in liquid nitrogen, and stored at −80°C.

**Flow cytometry and antibodies.** Single-cell suspensions were stained with fluorescent monoclonal antibodies against B220 (RA3-6B2), CD21 (7G6), CD23 (B3B4), CD24 (M1/69), IgM (DS-1), IgM (AF6-78), CD90.1/Thy1.1 (OX-7), and CD1d (1B1) purchased from either BD or eBioscience. Antibodies against IgM (R33-24.12; Leptin et al., 1984), IgD (1.3-5; Roes et al., 1995), CD19 (1D3; Kroop et al., 1996), B1-8Hi (Ac-146; Keth et al., 1979), and Bcl-2 (3F11; a gift of P. Marrack; Reed et al., 1987) were generated in house. Biotinylated antibodies were revealed with fluorescein-conjugated streptavidin (Invitrogen). Bcl-2 (and isotype control) staining was performed in saponin-treated cells as previously described (Vezis et al., 1993). 1.25 µg/ml propidium iodide (PI) was added to some samples to exclude dead cells. Analysis was performed on live B220+ or CD19+ lymphocytes based on incorporation of PI and/or forward and side scatter using the Cyan analyzer (Dako) and Flowjo software (Tree Star, Inc.).

**pErk flow cytometric analysis.** Freshly isolated single-cell suspensions were rested on ice for 1 h in HBSS with Ca2+ and Mg2+ (Cellgro) containing 1% FBS. Cells were treated with 60 µM sodium pervanadate for 5 min at 37°C, fixed in 2% paraformaldehyde, and permeabilized in 90% methanol. Cells were washed with PBS and stained with 1 µg/ml anti-pErk Thr202/Tyr204 (1:200; Cell Signaling Technology) or isotype control antibodies for 45 min at room temperature. Primary antibodies were revealed with a fluorescent anti–rabbit IgG antibody (SouthernBiotech). Sodium pervanadate was prepared by incubating a mixture of 900 µl water, 100 µl of 200 mM NaN3, and 330 µl of 30% H2O2 for 5 min at room temperature to yield a 6-mM solution, and was used at a final dilution of 1:100.

**Generation of bone marrow chimera and retrogenic mice.** Recipient mice were irradiated as previously described (Velez et al., 2007). Donor bone marrow cells were mixed at the ratios indicated in the figures, and a total of 1−2 × 106 cells in 100 µl PBS were injected in a tail vein of each recipient. When generating retrogenic mice, donor mice were injected i.p. with 3.75 mg 5-FU 4 d before bone marrow harvest. Cells were cultured...
overnight at 10^6 cells/ml in complete IMDM (100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 mM β-mercaptoethanol, 2 mM Glutamax, 0.1 mM of nonessential amino acids, 10% heat-inactivated FBS; Invitrogen) supplemented with recombinant cytokines (IL-3, IL-6, and stem cell factor; a gift of Y. Refaeli, NJH, Denver, CO). After 24 h, the cells were resuspended in 0.5 ml of complete IMDM and were added to 1 ml of retroviral supernatant, 3.2 µg (2.1 µg/ml final concentration) polybrevine, and recombinant cytokines, and centrifuged at 1,124 g for 1.5 h at room temperature. Cells were cultured overnight in fresh complete medium containing recombinant cytokines and, most times, transduced a second time as described. Recipient mice received a total of 1–5 x 10^6 cells via tail vein injection. Mice were analyzed 5–8 wk later except where indicated in the figures.

In vitro and in vivo B cell survival assays. Spleen mature B cells were enriched by magnetic bead depletion of CD43^+ cells on an AutoMACS (Miltenyi Biotech) according to the manufacturer's protocol. For in vitro cell survival, B cells were cultured overnight in complete RPMI 1640 medium to remove the effect of endogenous cytokines. The next day, viable cells were purified over Ficoll-Paque Premium (GE Healthcare) and were plated at 10^6 cells/ml in complete RPMI 1640 medium with or without 20 ng/ml of recombinant mouse BAFF (R&D Systems). Viable cells were enumerated by Trypan blue exclusion on subsequent days. For in vivo cell survival, pooled B cells isolated from groups of 3-83Ig^+ and 3-83Ig^-low mice were mixed 1:1 with pooled B cells from B1-8H3/3-83k mice. A total of ~30 x 10^6 cells were injected i.v. into sublethally irradiated (500 rad) B cell–deficient mice (n = 1–2 mice; Pelanda et al., 2002a). Irradiation was performed to allow for donor cell establishment (Baumgarth et al., 2000). A sample of each cell mixture was stained with anti-B1-8H3-38k (Ac-146), CD19, and IgM antibodies to determine the frequency of 3-83Ig^+ (B1-8H3-38k-negative) donor B cells before adoptive cell transfer (day 0). Recipient mice were euthanized 21 or 41 d after cell transfer, and spleen cells were stained and analyzed as on day 0.

In vitro B cell differentiation and immature B cell transduction. Bone marrow cells were cultured in complete IMDM in the presence of IL-7 for 3–4 d to enrich for IgM^+ immature B cells (Melamed et al., 1997). Cells were then washed twice with PBS to remove IL-7 and plated at 2–4 x 10^4 cells/ml with 10 ng/ml of recombinant mouse BAFF. On subsequent days, cells were stained with antibodies against B220, CD21, CD23, IgM, and IgD to determine the differentiation state. The MEK1/2 inhibitor PD98059 (10 µM) and the PI3-kinase inhibitor wortmannin (10 µM) were added to the culture medium to remove the effect of endogenous cytokines. The next day, viable cells were purified over Ficoll-Paque Premium (GE Healthcare) and were plated at 10^6 cells/ml in complete RPMI 1640 medium with or without 20 ng/ml of recombinant mouse BAFF (R&D Systems). Viable cells were enumerated by Trypan blue exclusion on subsequent days. For in vivo cell survival, pooled B cells isolated from groups of 3-83Ig^+ and 3-83Ig^-low mice were mixed 1:1 with pooled B cells from B1-8H3/3-83k mice. A total of ~30 x 10^6 cells were injected i.v. into sublethally irradiated (500 rad) B cell–deficient mice (n = 1–2 mice; Pelanda et al., 2002a). Irradiation was performed to allow for donor cell establishment (Baumgarth et al., 2000). A sample of each cell mixture was stained with anti-B1-8H3-38k (Ac-146), CD19, and IgM antibodies to determine the frequency of 3-83Ig^+ (B1-8H3-38k-negative) donor B cells before adoptive cell transfer (day 0). Recipient mice were euthanized 21 or 41 d after cell transfer, and spleen cells were stained and analyzed as on day 0.

Western blot analysis. Bone marrow cells from 3-83Ig^+ and 3-83Ig^-low mice were cultured in IL-7 for 4 d as described. Cells were lysed for 15 min on ice in a buffer containing 20 mM Tris-HCl, pH 8; 1% Triton X-100; 150 mM NaCl, 2 mM EDTA; 10% glycerol with 1 µg/ml each of leupeptin, aprotinin, and 0.1-antipyrinase; 1 mM Na_2VO_3; and 1 mM NaN_3. Lysates were cleared by centrifugation at 9,300 g, 10 min at 4°C and loaded on a 12% gel for denaturing SDS-PAGE. Proteins were transferred to nitrocellulose and probed with polyclonal rabbit anti-Igα (a gift from J. Cambier, NJH, Denver, CO) or monoclonal anti β-actin (I-19; Santa Cruz Biotechnology, Inc.) antibodies. Specific proteins were revealed with IRDye conjugated secondary antibodies (Rockland), and Igα protein band intensity was quantified after normalization to β-actin on the Odyssey Infrared Imaging System (LI-COR Biosciences).

Statistical analysis. Statistical significance was calculated with Prism software (GraphPad Software, Inc.) using a one-tailed Student’s t test with equal variance (using Welch’s correction when appropriate). P < 0.05 was considered significant. Data are represented as means ± SD.

Online supplemental material. Fig. S1 describes the generation of transitional and mature B cells in B1-8H3/3-83k-low mice. Fig. S2 describes the generation of mature B cells in BCR-low mouse with a normal Ig repertoire. Fig. S3 shows representative flow cytometric analysis of Bcl-2 expression in 3-83Ig^+ and 3-83Ig^-low bone marrow and spleen B cells. Fig. S4 shows the phenotypic characterization of B cells in IL-7–bone marrow cultures. Fig. S5 describes the differentiation of B1-8H3/3-83k-low immature B cells in vitro. Fig. S6 shows representative flow cytometric analysis of pErk expression in 3-83Ig^+ and 3-83Ig^-low bone marrow and spleen B cells. Fig. S7 describes the generation of mature B cells in 3-83Ig^-int mouse, and shows the in vitro differentiation of 3-83Ig^-int immature B cells. Table S1 displays the absolute bone marrow and spleen cell numbers recovered from bone marrow chimera mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091673/DC1.

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