Cellular Maturation Defects in Bruton's Tyrosine Kinase-Deficient Immature B Cells Are Amplified by Premature B Cell Receptor Expression and Reduced by Receptor Editing[1]

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In the mouse, Bruton's tyrosine kinase (Btk) is essential for efficient developmental progression of CD43^{+}CD2^{-} large cycling into CD43^{-}CD2^{+} small resting pre-B cells in the bone marrow and of IgM^{high} transitional type 2 B cells into IgM^{low} mature B cells in the spleen. In this study, we show that the impaired induction of cell surface changes in Btk-deficient pre-B cells was still noticeable in κ^{+} immature B cells, but was largely corrected in λ^{+} immature B cells. As λ gene rearrangements are programmed to follow κ rearrangements and λ expression is associated with receptor editing, we hypothesized that the transit time through the pre-B cell compartment or receptor editing may affect the extent of the cellular maturation defects in Btk-deficient B cells. To address this issue, we used 3-83$\mu\delta$ transgenic mice, which prematurely express a complete B cell receptor and therefore manifest accelerated B cell development. In Btk-deficient 3-83$\mu\delta$ mice, the IgM^{+} B cells in the bone marrow exhibited a very immature phenotype (pre-BCR^{+}CD43^{+}CD2^{-}) and were arrested at the transitional type 1 B cell stage upon arrival in the spleen. However, these cellular maturation defects were largely restored when Btk-deficient 3-83$\mu\delta$ B cells were on a centrally deleting background and therefore targeted for receptor editing. Providing an extended time window for developing B cells by enforced expression of the antiapoptotic gene *Bcl-2* did not alter the Btk dependence of their cellular maturation. We conclude that premature B cell receptor expression amplifies the cellular maturation defects in Btk-deficient B cells, while extensive receptor editing reduces these defects. *The Journal of Immunology,* **2004, 172: 1371–1379.**

The generation of B cells from progenitor cells is a complex process involving the transit of cells through several critical developmental stages. Developing B cell precursors in the bone marrow pass several checkpoints at which they are subject to choices between survival, proliferation, or cell death dependent on the presence of functional Ig H and L chains. Expression of the pre-B cell receptor (pre-BCR[3]) complex, comprised of μ H chain, the V_{preB} and λ5 surrogate L chain (SLC) proteins, and the Igα/CD79a and Igβ/CD79b signaling components, is essential for IL-7-dependent proliferative expansion of cytoplasmic μ H chain positive (cμ^{+}) pre-B cells (1, 2). In addition, the pre-BCR triggers the inhibition of further H chain rearrangements, known as allelic exclusion, as well as drastic changes of pre-B cell surface phenotype. Furthermore, the down-regulation of proliferation in large cycling pre-B cells and the induction of progression into small resting pre-B cells, in which Ig L chain rearrangement is initiated, are regulated by the pre-BCR. Signaling proteins involved in the downstream pathway of the pre-BCR include the Lyn, Syk, and Bruton's tyrosine kinase (Btk) tyrosine kinases, phosphoinositide 3-kinase (consisting of p85 and p110 subunits), the B cell linker protein BLNK/SLP-65 and the phospholipase Cγ2. Cytoplasmic μ^{+} pre-B cells of mice deficient for the pre-BCR components λ5, V_{preB}, membrane μ H chain, Igα, or Igβ or the pre-BCR signaling molecules Syk or p85α do not enter the expansion phase (reviewed in Ref. 3).

In contrast, absence of the pre-BCR signaling proteins Btk and BLNK/SLP-65 was shown to be associated with defects in cell cycle exit, resulting in a high incidence of pre-B cell tumors (4–8). In SLP-65-deficient mice, and even more so in Btk/SLP-65 double mutant mice, large cycling pre-B cells are trapped in a positive feedback loop as they are arrested in development and cannot efficiently down-regulate pre-BCR expression, which signals for proliferation (7–9). Mutations in the *Btk* gene result in X-linked agammaglobulinemia in humans and x-linked immunodeficiency (*xid*) in the mouse (10). X-linked agammaglobulinemia is characterized by a severe arrest of B cell development at the pre-B cell stage, and consequently peripheral B cells and Abs of all subclasses are almost completely absent. *xid* mice manifest a milder phenotype associated with an impairment of peripheral B cell maturation, without a major early block in B cell development in the bone marrow (BM). As a result, peripheral B cells are present in lower numbers and serum IgM and IgG3 levels are low.

Recently, we have shown that pre-B cell differentiation in Btk-deficient mice is impaired (5, 8). During the transition of large cycling into small resting pre-B cells, Btk-deficient mice fail to efficiently modulate the expression of SLC, the metallopeptidase BP-1, the adhesion molecule CD2, the IL-2R CD25, MHC class II, and the membrane sialoglycoprotein CD43. Moreover, in an analysis of the kinetics of pre-B cell differentiation in vivo, Btk-deficient cells manifested a specific developmental delay within the

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[3] Abbreviations used in this paper: BCR, B cell receptor; SLC, surrogate L chain; BM, bone marrow; Btk, Bruton's tyrosine kinase; Tg, transgenic; WT, wild type; cμ, cytoplasmic μ H chain; BrdU, 5-bromo-2-deoxyuridine; T1/2, transitional type 1 and 2.

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small pre-B cell compartment of ~3 h when compared with wild type (WT) cells. Despite the similar absolute numbers of immature B cells generated in both mice, Btk^- immature B cells are phenotypically different from WT immature B cells. The impaired induction of cell surface changes in Btk-deficient small pre-B cells is still noticeable at the immature B cell stage, in particular with regard to the expression levels of BP-1 and CD2 (5). In this respect, we now found that κ L chain-positive immature B cells are more affected by the lack of Btk than λ L chain-positive immature B cells. Given that λ gene rearrangements in pre-B cells are programmed to occur ~24 h later than κ rearrangements, it is possible that the transit time through the pre-B cell compartment may affect the differentiation defects in Btk-deficient B cells. Alternatively, as λ L chain expression is often associated with receptor editing, the BCR signaling events that induce receptor editing may also facilitate cellular maturation.

To distinguish between these possibilities, we modified the kinetics of B cell development by crossing Btk-deficient mice with 3-83$\mu\delta$-transgenic (Tg) mice, which carry a BCR transgene consisting of prerearranged μ and δ H chain genes as well as a κ L chain gene (11). Because the expression of the 3-83$\mu\delta$ transgene is under the control of the Ig H chain enhancer, the 3-83$\mu\delta$ BCR is expressed from the pro-B cell stage onward. The 3-83$\mu\delta$ BCR specifically recognizes MHC class I molecules of the $H2\text{-}K^{k/b}$ allotype (11), and thus reflects an innocuous BCR in mice that have an $H2\text{-}K^d$ background. Mice with such a nonautoreactive background (also called nondeleting mice) contain a virtually monoclonal B cell population. Since the prematurely expressed 3-83$\mu\delta$ BCR is able to promote B cell development, it appears that this BCR can functionally replace the pre-BCR and consequently B cell development is accelerated (11–13). In contrast, when the 3-83$\mu\delta$ BCR is expressed in mice of the MHC class I $H2\text{-}K^b$ background, all B cells generated are autoreactive and are targeted for continued L chain gene rearrangement, a process known as receptor editing, to survive (14–16). B cells in these centrally deleting mice are delayed in the small pre-B cell stage due to the receptor editing process (17, 18).

By crossing Btk-deficient mice with 3-83$\mu\delta$ Tg mice on a nondeleting or a deleting MHC class I background, we were able to study the effects of Btk on cellular maturation of B cell precursors undergoing accelerated development or receptor editing, respectively. Furthermore, we tested whether providing an extended time window per cell for the induction of cell surface marker changes, by the enforced expression of the Bcl-2 apoptosis inhibitor, would facilitate cellular maturation in Btk-deficient pre-B cells.

Materials and Methods

Mice and genotyping

Btk-deficient mice (19) were crossed onto the C57BL/6 background for more than eight generations. 3-83$\mu\delta$ mice (11) were on a nondeleting B10.D2 background. Endogenous Btk WT alleles were identified as described previously (5). Expression of a 3-83$\mu\delta$ was identified by a 3-83 $V\kappa$ forward primer (5′-CAGCTTCCTGCTAATCAGTGCC-3′) and a 3-83 $J\kappa2$ reverse primer (5′-TGGTCCCCCCTCCGAACGTG-3′) (11). MHC I background was determined by FACS analysis of peripheral blood samples by using mAbs against $H2\text{-}K^b$ and $H2\text{-}K^d$ (below). The presence of the *Eμ-2–22 Bcl-2* transgene was evaluated by PCR (20).

Flow cytometric analysis

Preparations of single-cell suspensions, standard and intracellular flow cytometry, and determination of β-galactosidase activity by loading cells with fluorescein-β-D-galactopyranoside substrate have been described previously (5, 19, 21). The hybridomas LM34 (anti-SLC; (22)) and PB493 (anti-AA4.1; (23)) were kindly provided by A. Rolink (University of Basel, Basel, Switzerland). The anti-3-83$\mu\delta$ hybridoma 54-1 (15) was kindly provided by D. Nemazee (The Scripps Research Institute, La Jolla, CA). Hybridoma Abs were purified using protein G columns and conjugated to biotin according to standard methods or used unlabeled in combination with PE- or allophycocyanin-conjugated goat anti-rat secondary Abs. The following mAbs were obtained from BD PharMingen (San Diego, CA): FITC-conjugated anti-CD21 (7G6), anti-CD22 (Cy34.1), and anti-CD62L (MEL-14); PE-conjugated anti-MHC class II (M5/114), anti-CD19 (1D3), anti-IgM (R6-60.2), anti-CD23 (B3B4), and anti-$H2\text{-}K^d$ (SF1–1.1); PerCP- and allophycocyanin-conjugated anti-B220 (RA3-6B2) and anti-CD19 (1D3); biotinylated anti-Ly6C (ER-MP20), anti-CD23 (B3B4), and anti-$H2\text{-}K^b$ (AF6-88.5). Unlabeled anti-CD21 (7G6; BD PharMingen) was biotinylated according to standard methods and FITC-conjugated anti-IgD (11–26) was obtained from Southern Biotechnology Associates (Birmingham, AL). In vivo 5′-bromo-2′deoxyuridine (BrdU) labeling and detection and all other mAbs used were described previously (5).

IL-7-driven BM cultures

Primary pre-B cell BM cultures and determination of IL-7-dependent proliferative responses of total BM cells have been described previously (5, 20).

Results

In Btk-deficient mice, κ^+ and λ^+ immature B cells show differences in cellular maturation

During the progression of large cycling into small resting cμ H chain-positive pre-B cells, Btk-deficient mice manifest defective down-regulation of BP-1, CD43, and SLC and impaired up-regulation of CD2, CD25, and MHC class II, as shown by flow cytometric analysis (Fig. 1, *A* and *B* and Refs. 5 and 8). In this study, we have also included the mAb PB493 recognizing the complement component C1q-like receptor C1qRp, also known as AA4.1 (24). This marker is expressed on all B lineage cells in the BM, but in the spleen only on those B cells that reflect recently immigrated immature B cells (23). In Btk-deficient mice, we found increased proportions of $PB493^+$ cells in the $B220^+IgM^-$ B cell precursor compartment when compared with Btk^+ mice (Fig. 1*B*), indicating that the modulation of expression of this marker is also Btk dependent. As shown in Fig. 1*B*, the proportions of cells that express μ H chain in their cytoplasm within the surface IgM^- B cell precursor fractions are similar in Btk-deficient and WT mice.

We previously found that the inefficient induction of cell surface phenotype changes in Btk-deficient mice was still noticeable in immature B cells, in particular for BP-1 and CD2 (5). In a separate analysis of κ and λ L chain-positive $B220^{low}IgM^+$ immature B cells, we now observed that elevated expression of BP-1 was more pronounced in Btk-deficient κ^+ immature B cells when compared with λ^+ cells. Reduced expression of CD2, MHC class II and CD25 was limited to κ^+ immature B cells, while λ^+ immature B cells manifested essentially normal surface expression of these markers (Fig. 1*C* and data not shown).

In summary, these findings indicate that in Btk-deficient mice the impaired modulation of developmentally regulated markers in small pre-B cells results in very immature phenotype of κ^+ B cells in the BM, while λ^+ B cells appear less affected.

Btk-deficient 3-83$\mu\delta$ mice show amplified B cell differentiation defects

We hypothesized that the observed differences in surface phenotype between Btk-deficient κ^+ and λ^+ immature B cells may reflect the programmed sequential activation of the κ and λ loci. In pre-B cells, λ gene rearrangements are programmed to occur ~24 h later than κ rearrangements, and thus λ^+ immature B cells have resided for a longer period of time within the small pre-B cell compartment (25, 26). Therefore, the extent of the differentiation defects in Btk-deficient B cells may correlate with the rate of transit through the pre-B cell compartment. To directly test this hypothesis, we crossed Btk-deficient mice with 3-83$\mu\delta$ Tg mice, which prematurely express a prerecombined BCR that functionally replaces the pre-BCR. As a result, B cells spend little or no time in

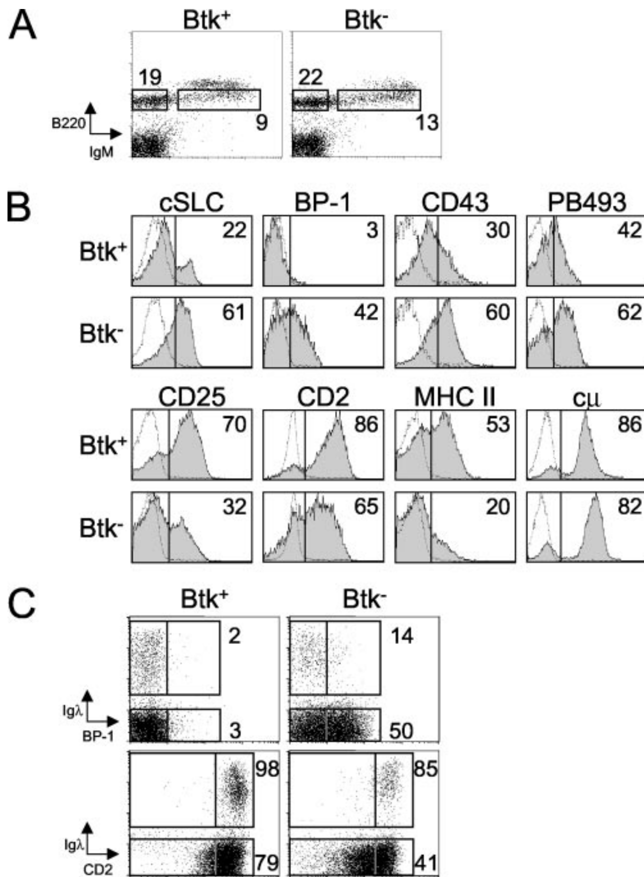

FIGURE 1. Phenotypic characterization of the B lineage compartment in BM of Btk$^+$ and Btk$^-$ mice. *A,* BM lymphoid cells were electronically gated on the basis of forward and side scatter and expression of B220 vs IgM is plotted. *B,* B220^{low}IgM$^-$ pro- and pre-B cells were gated and expression of several developmentally regulated markers is shown as histograms. Numbers indicate percentage of cells positive for the indicated marker and plots are representative for ~20 mice of each genotype. *C,* B220^{low}IgM$^+$ immature B cells were gated and expression of Igλ and BP-1 or CD2 is shown. Numbers indicate percentage of BP-1- or CD2-positive cells within λ^+ or κ^+ immature B cells. Plots are representative for four mice of each genotype.

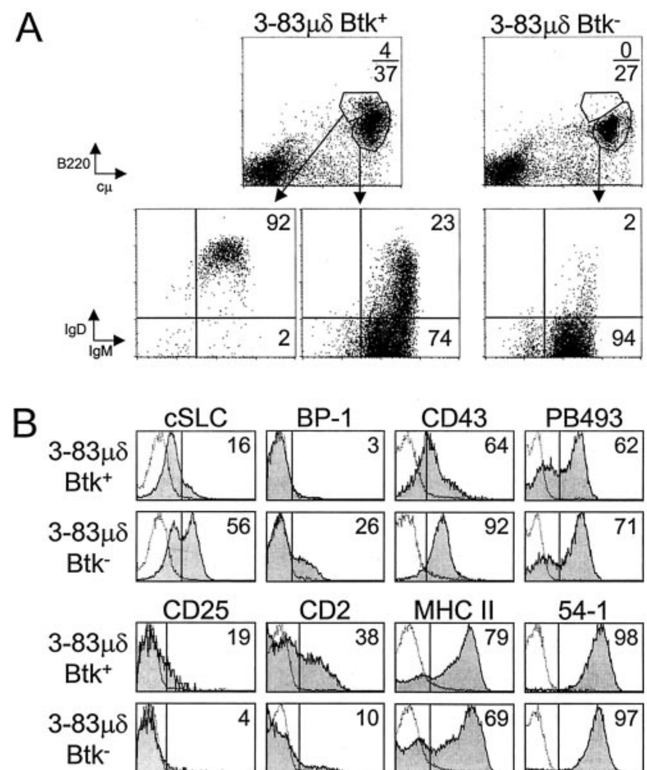

FIGURE 2. Phenotypic characterization of the BM B cell compartment in Btk$^+$ and Btk$^-$ 3-83$\mu\delta$ mice. *A,* BM lymphoid cells were gated and plotted for B220 and cμ (*upper part*). B220^{high} and B220^{low} cells were separately gated and IgM/IgD profiles of both populations are shown (*lower part*). *B,* The B220^{low} B cell population was further analyzed for expression of various developmentally regulated markers, as shown in the histograms. The percentages of cells within the indicated gates or quadrants are given. Data represent six to eight mice analyzed per genotype.

the small pre-B cell stage and B cell development is accelerated significantly.

Total BM cell suspensions from Btk$^+$ and Btk$^-$ 3-83$\mu\delta$ mice were analyzed by flow cytometry (Fig. 2). Irrespective of the presence of Btk, the B lineage compartment in 3-83$\mu\delta$ mice mainly consisted of B220^{low} immature B cells that were cμ^+ and also expressed IgM on the cell surface. However, the B220^{low} immature B cell fraction present in Btk$^-$ 3-83$\mu\delta$ mice contained very few IgD$^+$ B cells (<2%) when compared with Btk$^+$ 3-83$\mu\delta$ mice (23% IgD$^+$ cells; Fig. 2*A*). Furthermore, the BM of Btk$^-$ 3-83$\mu\delta$ mice did not contain mature B220^{high}IgD^{high} recirculating B cells. Next, the expression pattern of several developmental markers was analyzed in the B220^{low}IgM$^+$ immature B cell compartment. In WT mice, immature B cells did not express SLC, BP-1, and CD43, were low for PB493 and CD25, and positive for CD2 and MHC class II (Fig. 1*C* and Ref. 5). However, due to the expression of the 3-83$\mu\delta$ transgene in WT mice, the B220^{low}IgM$^+$ compartment had a more immature phenotype: CD43$^{+/low}$, PB493^{high}, CD25^{low}, and CD2^{low}. Notably, Btk-deficient 3-83$\mu\delta$ mice showed significantly impaired B cell maturation, as the B220^{low}IgM$^+$ B cells expressed substantial levels of CD43 and SLC and lacked expression of CD2 and CD25 (Fig. 2*B*), when compared with their Btk$^+$ 3-83$\mu\delta$ littermates. The expression of MHC class II on B220^{low}IgM$^+$ B cells did not appear to be affected by the premature BCR expression.

These results indicate that a prematurely expressed 3-83$\mu\delta$ BCR was only partially able to functionally replace the pre-BCR, as the modulation of expression of developmentally regulated markers was impaired. In Btk-deficient 3-83$\mu\delta$ mice, this defect was even stronger, as evidenced by an almost complete absence of down-regulation of CD43 and SLC and induction of CD2 and CD25 in IgM$^+$ B cells in the BM.

Btk-deficient 3-83μδ mice manifest a severe reduction of B cell numbers in the spleen

Total splenic cell suspensions from Btk$^+$ and Btk$^-$ 3-83$\mu\delta$ mice were analyzed by flow cytometry and compared with non-Tg littermates. The *xid* phenotype of Btk-deficient mice is characterized by a specific deficiency of mature IgM^{low}IgD^{high} B cells in the spleen, resulting in a reduction of the B cell numbers by ~50% (19, 27, 28). As previously described (16), expression of the 3-83$\mu\delta$ transgene on a WT background only marginally affected the splenic B cell numbers. Surprisingly, we observed a profound reduction of the size of the splenic B cell population in Btk$^-$ 3-83$\mu\delta$ mice, as $<2 \times 10^6$ B cells were present compared with $\sim 20 \times 10^6$ B cells in Btk$^+$ 3-83$\mu\delta$ mice (Fig. 3*A* and Table I).

This severe reduction of splenic B cells in Btk$^-$ 3-83$\mu\delta$ mice may reflect a reduced survival capacity of Btk$^-$ 3-83$\mu\delta$ B cells in the BM or in the spleen. Alternatively, it is possible that the absence of Btk affects the signals transmitted by the 3-83$\mu\delta$ BCR in

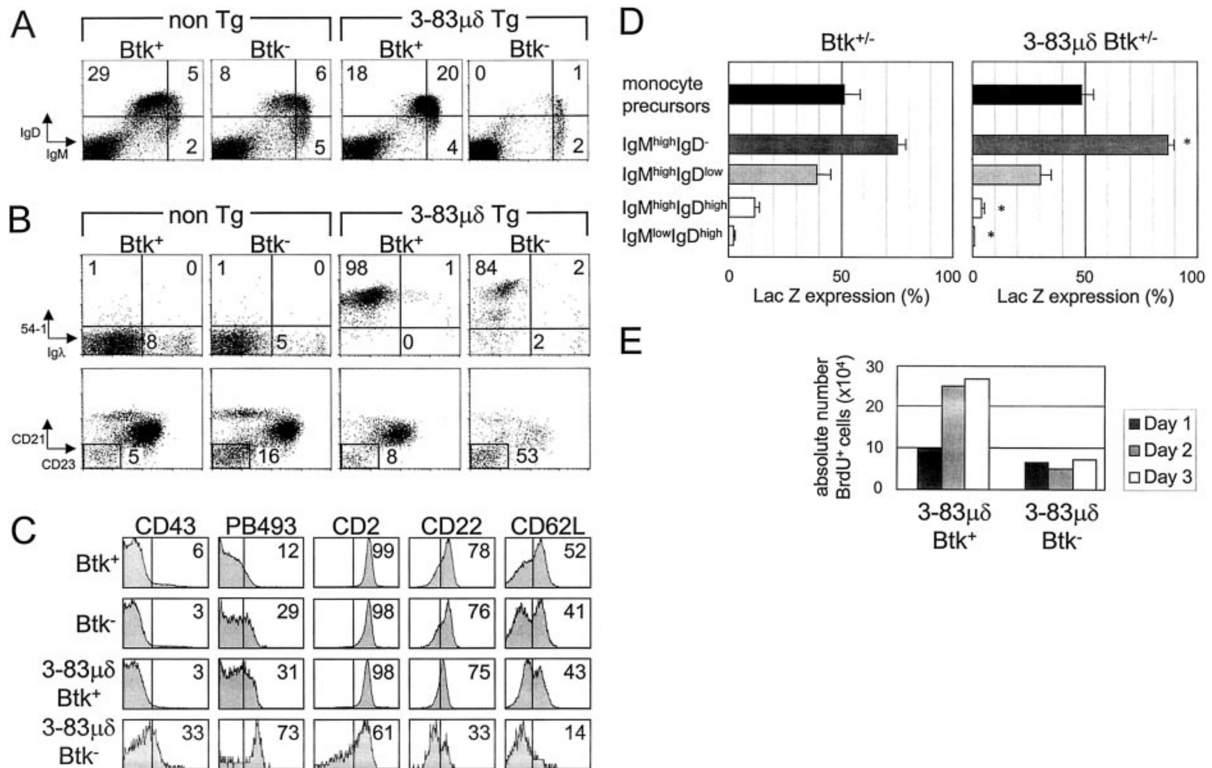

FIGURE 3. Phenotypic characterization of B cell compartments in spleens of Btk^+ and Btk^- non-Tg and 3-83$\mu\delta$ mice. *A,* Splenic lymphoid cells were gated and IgM/IgD expression profiles are plotted. *B,* $B220^+$ cells were gated and analyzed for expression profiles of 54-1/Igλ (*upper part*) and CD21/CD23 (*lower part*). *C,* $B220^+$ cells were gated and analyzed for the expression of CD43 and CD2 on IgM^+ cells and expression of PB493, CD22, and CD62L on $CD19^+$ cells. *A–C,* The percentages of cells within the indicated gates or quadrants are given; data represent three to eight mice analyzed per genotype. *D,* Expression of *lacZ* in splenic B cells of $Btk^{+/-}$ non-Tg and 3-83$\mu\delta$ heterozygous female mice. Splenic B cells were gated using IgM and IgD expression profiles, and *LacZ* expression in these populations was compared with *lacZ* expression in ER-MP20^{high} BM monocyte precursor cells. Mean percentages ± SD values of *lacZ*-positive cells within the indicated gates were plotted and significance between non-Tg and 3-83$\mu\delta$ mice was evaluated by Student's *t* test (*, $p \le 0.01$). Data represent four to five mice analyzed per genotype. *E,* BrdU incorporation in spleen after a single dose of BrdU injection in Btk^+ and Btk^- 3-83$\mu\delta$ mice. The absolute number of $BrdU^+$ cells was calculated within the $B220^+IgM^{high}IgD^{-/low}$ B cell population. Mean values of two to six mice per time point were plotted.

such a way that these B cells are considered autoreactive and are therefore deleted. Autoreactive B cells down-regulate surface IgM expression and are subject to receptor editing at the immature B cell stage in the BM (12, 14, 16, 29). However, flow cytometric analyses did not provide evidence for autoreactive characteristics of Btk^- 3-83$\mu\delta$ B cells. The B cells were IgM^{high} and did not edit their BCR, as they were mainly positive for the anti-idiotypic Ab 54-1 and κ L chain, both in BM and in spleen (Figs. 2, *A* and *B*, and 3, *A* and *B*).

The residual B cell population in Btk^- 3-83$\mu\delta$ spleens mainly consisted of immature $IgM^{high}IgD^{-/low}$ that were $CD21^-$ and $CD23^-$, indicating that these B cells were recent immigrants from the BM arrested at the stage of transitional type 1 (T1) B cells (Fig. 3*B* and Table I; Refs. 30 and 31). This was confirmed by an evaluation of additional developmentally regulated cell surface markers. As shown in Fig. 3*C*, splenic B cells in Btk^+ and Btk^- non-Tg mice and in Btk^+ 3-83$\mu\delta$ mice are largely $CD43^-$, $PB493^-$, CD2^{high}, CD22^{high}, and $CD62L^+$. In contrast, Btk^- 3-83$\mu\delta$ B cells showed significantly increased expression of the immature markers CD43 and PB493 and reduced expression of the maturation markers CD2, CD22, and CD62L. Taken together, only a small number of peripheral B cells reached the stage of T2 cells (IgM^{high}, IgD^+, $CD21^+$, $CD23^+$; Refs. 30 and 31) and mature IgM^{low} IgD^+ cells were virtually absent (Fig. 3 and Table I). The expression levels of SLC, BP-1, CD25, and MHC class II were similar in the four groups of mice (data not shown). Despite the low B cell numbers and their immature phenotype, splenic architecture was not seriously disturbed in Btk^- 3-83$\mu\delta$ mice as B/T cell separation was normal and small regions with marginal zone B cells and macrophages were present, as analyzed by immunohistochemistry (data not shown).

In summary, these findings show that peripheral Btk^+ 3-83$\mu\delta$ B cells differentiated normally. By contrast, Btk-deficient 3-83$\mu\delta$ mice exhibited a severe reduction of B cell numbers in the spleen, whereby the residual B cells present were Id positive and mainly had an immature T1 surface phenotype.

Btk-deficient 3-83μδ B cells are rapidly deleted upon arrival in the spleen

The finding that the proportions of $B220^+$ B lineage cells in BM of Btk^+ 3-83$\mu\delta$ mice were not significantly different from those in Btk^- 3-83$\mu\delta$ mice (5.5% ± 1.2 and 4.5% ± 1.8, respectively) implied that the numbers of B cells generated in the BM of Btk^+ and Btk^- 3-83$\mu\delta$ mice were comparable. Therefore, the severe decrease in splenic B cell numbers in Btk^- 3-83$\mu\delta$ mice may be caused by defective homing of immature B cells from the BM into the spleen or alternatively by rapid deletion upon arrival in the spleen. To distinguish between these two possibilities, we investigated the competition between Btk^+ and Btk^- 3-83$\mu\delta$ B cells in vivo. We analyzed heterozygous $Btk^{+/-}$ female mice, which have the *Btk*$^-$*lacZ*$^+$ allele on the active X chromosome in ~50% of all cells, due to random X chromosome inactivation. However, when

Table I. *Absolute number of B cell subsets in spleen*

Mouse Strain	N	B220+[a]	p[b]	IgM^high IgD^−/low (T1)[c]	IgM^high IgD^high (T2)	IgM^low IgD^high (Mature)
Btk+	17	37.8 ± 2.4		2.1 ± 0.2	5.5 ± 0.5	25.0 ± 2.0
Btk−	15	16.1 ± 1.5	$<1 \times 10^{-7}$	2.2 ± 0.3	5.5 ± 0.8	5.8 ± 0.6
3-83μδ Btk+ nondeleting	19	16.0 ± 1.2		1.3 ± 0.2	6.3 ± 0.8	6.9 ± 0.6
3-83μδ Btk− nondeleting	14	1.2 ± 0.1	$<1 \times 10^{-10}$	0.5 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
3-83μδ Btk+ deleting	4	4.0 ± 1.4		0.7 ± 0.3	1.0 ± 0.4	1.8 ± 0.8
3-83μδ Btk− deleting	7	2.1 ± 1.0	NS	0.2 ± 0.1	0.4 ± 0.2	1.0 ± 0.6
3-83μδ/Bcl-2 double Tg Btk+ nondeleting	3	28.3 ± 2.8		0.7 ± 0.2	11.7 ± 0.8	11.3 ± 2.6
3-83μδ/Bcl-2 double Tg Btk− nondeleting	3	15.8 ± 4.1	<0.05	5.3 ± 1.3	6.0 ± 1.3	1.9 ± 0.7

[a] Absolute number as mean ± SEM ($\times 10^6$).
[b] Values of P for the difference in absolute numbers of B220+ cells compared with Btk+ littermates, determined by Student's t test.
[c] T1, T2, and mature B cell subsets of B220+ cells.

in these mice B lineage cells reach a differentiation stage in which Btk is required, the *Btk acZ*+ cells will be arrested in development and consequently the proportions of *Btk lacZ*+ cells decrease below 50% (19).

BM and spleen cell suspensions from $Btk^{+/-}$ non-Tg and 3-83μδ mice were compared for the proportions of *lacZ*-expressing cells by using fluorescein-di-β-galactopyranoside as a substrate in conjunction with surface labeling of B220, IgM, and IgD. Irrespective of the presence of the 3-83μδ transgene, the spleens of $Btk^{+/-}$ mice contained detectable fractions of *lacZ*+ B cells, indicating that homing of B cells from the BM into the spleen was not altered by the expression of the 3-83μδ BCR (Fig. 3*D*). Moreover, the immature IgM+IgD− fraction in 3-83μδ mice contained >50% *lacZ*+ cells, showing that Btk-deficient B cells accumulated due to the differentiation arrest. Consistent with previous findings (19), a dramatic selection against *Btk*−*lacZ*+ cells occurred during B cell differentiation in the spleen: the immature $IgM^+IgD^{-/low}$ cell populations still contained substantial proportions of *Btk*−*lacZ*+ cells, whereas in the $IgM^{low}IgD^{high}$ mature B cell population these cells were virtually absent (Fig. 3*D*).

To confirm that Btk-deficient 3-83μδ B cells were deleted upon arrival in the spleen, we performed BrdU-labeling experiments in vivo. Btk+ or Btk− 3-83μδ mice were injected with a single dose of BrdU, which is incorporated into the DNA of large cycling pre-B cells in the BM (17, 25, 32). The amount of BrdU incorporation was found to be similar in BM B cells from both types of mice (data not shown). One, 2, or 3 days after BrdU injection, spleen cell suspensions were stained for B220, IgM, and IgD in conjunction with intracellular staining for BrdU. $B220^+IgM^{high}IgD^{-/low}$ cells were gated and the absolute number of BrdU+ cells was calculated. We found that in Btk+ 3-83μδ mice the number of $BrdU^+IgM^{high}IgD^{-/low}$ B cells accumulated over time (Fig. 3*E*). In contrast, the number of BrdU+ cells in Btk− 3-83μδ mice remained low, indicating that the number of cells arriving in the spleen was similar to the number of cells that was deleted.

From these findings, we conclude that Btk− 3-83μδ immature B cells migrated normally from the BM into the spleen, but upon arrival these B cells did not further differentiate and were deleted within 24 h.

Btk− 3-83μδ BM B cells show deficient developmental progression in vitro

To investigate whether the inefficient 3-83μδ BCR-mediated induction of cell phenotype changes in Btk− B cells in vivo were paralleled in BM cultures in vitro, IL-7-driven BM cultures were performed as described previously (5, 33). Total BM cells from Btk+ and Btk− non-Tg and 3-83μδ mice were cultured in the presence of 100 U/ml IL-7 for 5 days and proliferation was quantified in [^{3}H]thymidine incorporation experiments (Fig. 4*A*). Consistent with our previous report (5), Btk− non-Tg BM cultures showed significantly higher proliferative responses to IL-7 when compared with Btk+ cultures. Proliferative responses to IL-7 were similar in Btk+ and Btk− 3-83μδ BM cultures. To evaluate developmental progression in vitro, BM cells were cultured in the presence of IL-7 for 5 days and recultured in the absence of IL-7 on S17 stromal cells for 48 h. As previously described (5), under these conditions, WT pre-B cells differentiated into immature B cells expressing CD2, IgM, and IgD, whereas Btk-deficient pre-B cells were not able to efficiently up-regulate CD2 and IgD expression (Fig. 4*B*). In BM cultures from Btk+ 3-83μδ mice, expression of CD2 and IgD was induced on the B cells, but Btk− 3-83μδ B cells were not able to up-regulate CD2 and IgD upon IL-7 withdrawal (Fig. 4*B*).

These data show that also under in vitro conditions, developmental progression of Btk− 3-83μδ BCR-expressing B cells is

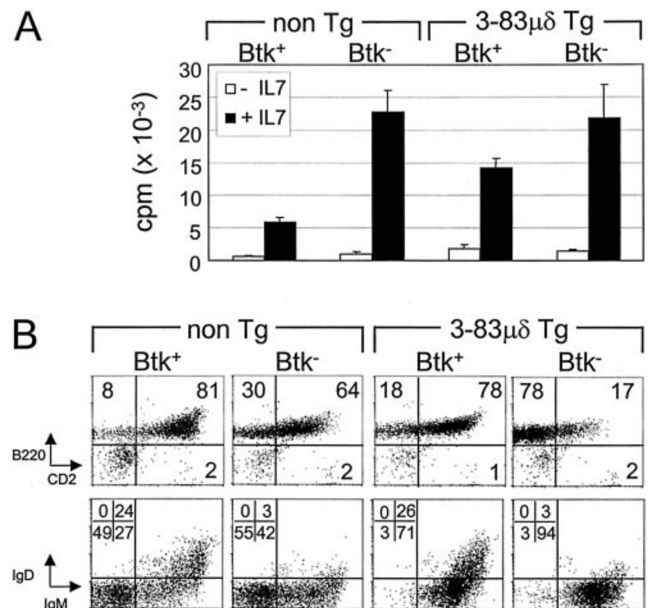

FIGURE 4. Analysis of IL-7-driven BM cultures from Btk+ and Btk− non-Tg and 3-83μδ mice. *A,* Proliferative responses of total BM cells as determined by [^{3}H]thymidine incorporation after 5 days of culture with or without IL-7. Means ± SD values for two to four mice per genotype were plotted. *B,* After 5 days of culture with IL-7, cells were washed and recultured for 48 h on S17 stromal cells in the absence of IL-7. Expression profiles of B220/CD2 on live cells (*upper part*) and of IgM/IgD on B220+ gated cells (*lower part*) are shown. Data are representative of five mice analyzed per group.

impaired, as is clear from low CD2 and IgD induction upon IL-7 withdrawal.

Receptor editing can compensate for developmental defects in Btk⁻ 3-83μδ mice

To investigate whether receptor editing and the resulting delay at the small pre-B cell stage in the BM could compensate for the cellular maturation defects in Btk^- 3-83$\mu\delta$ mice, we targeted 3-83$\mu\delta$ B cells for receptor editing by crossing the 3-83$\mu\delta$ mice onto a centrally deleting MHC class I background.

When we analyzed the BM of Btk^+ and Btk^- 3-83$\mu\delta$ centrally deleting mice by flow cytometry, we found that $B220^+c\mu^+$ cells had down-regulated surface expression of IgM and CD19 and were also low for the anti-idiotypic Ab 54-1, confirming the autoreactive nature of the B cell compartment (Fig. 5*A*). The expression profiles of developmentally regulated surface markers on $B220^{low}c\mu^+$ cells in Btk^+ 3-83$\mu\delta$ mice were similar to those found in non-Tg WT immature B cells, i.e., $SLC^-CD43^-CD2^+$ (Fig. 5*B*). Remarkably, Btk^- 3-83$\mu\delta$ centrally deleting mice manifested an almost complete correction of marker expression profiles when compared with Btk^- 3-83$\mu\delta$ nondeleting mice: expression of SLC and CD43 was low and CD2 was present on a large fraction of cells (cf Figs. 5*B* and 2*B*). Therefore, we conclude that extended receptor editing in the BM could almost completely correct the Btk-dependent defects in B cell surface marker expression of 3-83$\mu\delta$ immature B cells in the BM.

The spleens of 3-83$\mu\delta$-deleting mice contained very few B cells, irrespective of the presence of Btk (Fig. 5*C* and Table I). As a result, the total splenic B cell numbers in Btk^- 3-83$\mu\delta$ mice were comparable on the nondeleting ($\sim 1.2 \times 10^6$) and the deleting MHC class I background ($\sim 2.1 \times 10^6$; Table I). Those Btk^+ and Btk^- B cells present in the spleen of 3-83$\mu\delta$-deleting mice have essentially lost 54-1 expression and show increased Ig λ L chain usage due to receptor editing (cf Figs. 5*C* and 3*B*). Remarkably, we found that the residual B cells in the spleens of Btk^+ and Btk^- 3-83$\mu\delta$-deleting mice had similar IgM/IgD and CD21/CD23 profiles (Fig. 5*C*), had similar expression levels of CD43, CD2, CD19, and MHC class II, and were negative for SLC, BP-1, and CD25 (Fig. 5*D* and data not shown).

In summary, these data indicate that both Btk^+ and Btk^- 3-83$\mu\delta$ B cells that were targeted for receptor editing have reduced maturation defects when compared with Btk^+ and Btk^- 3-83$\mu\delta$ B cells on a nondeleting background. The expression of the developmentally regulated markers is completely corrected in edited Btk^+ 3-83$\mu\delta$ peripheral B cells, while in edited Btk^- 3-83$\mu\delta$ cells the cellular maturation defects are comparable to those found in non-Tg Btk^- B cells (Figs. 1 and 2).

Bcl-2 overexpression does not rescue Btk-dependent marker modulation

We investigated whether the observed correction of marker modulation in editing Btk-deficient pre-B cells was dependent on additional signals that follow the engagement of an autoreactive BCR with autoantigen or alternatively resulted from the developmental delay in the small pre-B cell stage associated with receptor editing. To this end, Btk-deficient mice were crossed with Tg mice that express the antiapoptotic *Bcl-2* gene, which is assumed to provide an extended time window per cell for Ig L chain rearrangement (34). When we compared the B cell compartment in Btk^+ and Btk^- Eμ-Bcl-2 Tg mice, we found that enforced expression of Bcl-2 only slightly improved cellular maturation of pre-B and immature B cells in the absence of Btk (cf Figs. 6, *A* and *B*, and 1).

Next, we investigated whether Bcl-2 expression could rescue the developmental defects observed in Btk^- 3-83$\mu\delta$ nondeleting mice.

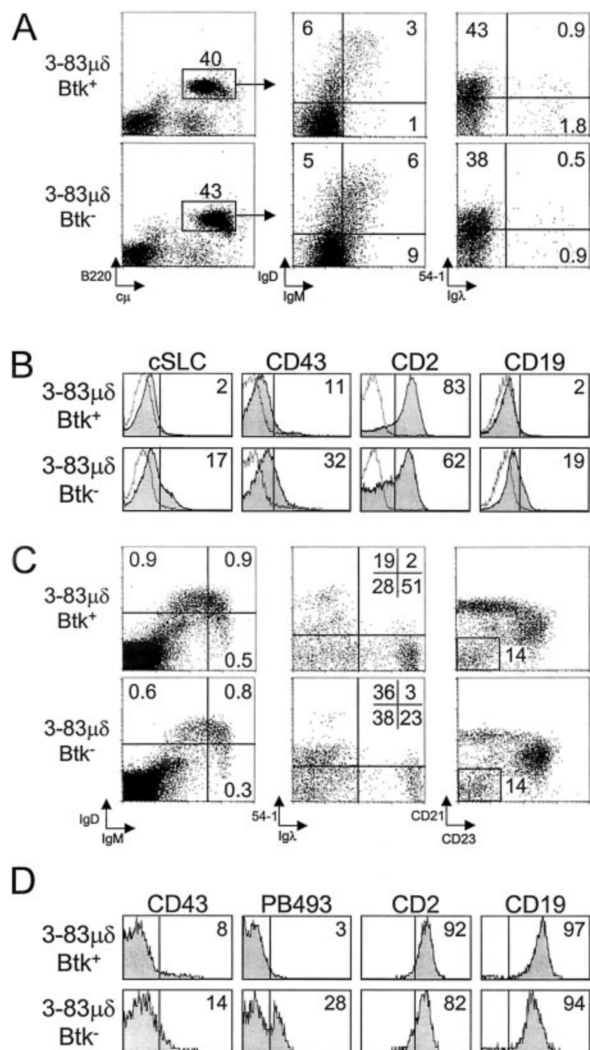

FIGURE 5. Phenotypic characterization of the B cell compartment in Btk^+ and Btk^- 3-83$\mu\delta$ mice on a centrally deleting background. *A* and *B*, BM lymphoid cells were gated and plotted for the expression of B220 and cμ. $B220^+c\mu^+$ cells were gated and plotted for IgM/IgD and 54-1/Igλ expression profiles (*A*) and analyzed for the expression of the indicated markers (*B*). *C*, Splenic lymphoid cells were gated and analyzed for IgM/IgD expression and for CD21/CD23 and 54-1/Igλ expression profiles on $B220^+$ gated cells. *D*, Expression pattern of the indicated markers on $B220^+$ cells. The percentages of cells within the indicated gates or quadrants are given. Data represent 8–20 mice analyzed per genotype.

Therefore, Btk-deficient 3-83$\mu\delta$ mice were crossed with Eμ-Bcl-2 Tg mice on a non-deleting MHC class I background. Overexpression of Bcl-2 did not rescue cellular maturation of Btk^- 3-83$\mu\delta$ immature B cells, as expression of SLC, BP-1, CD43, and PB493 was not down-regulated and expression of CD25, CD2, and IgD was hardly induced (Fig. 6*C* and data not shown). The BM B cells in Btk^+ and Btk^- 3-83$\mu\delta$/Bcl-2 double Tg mice were all 54-1^+ (data not shown) and did not show increased λ usage ($<0.3\%$). The absolute number of splenic B cells in Btk^- 3-83$\mu\delta$/Bcl-2 double Tg mice was corrected when compared with Btk^- 3-83$\mu\delta$ mice and the B cells in the spleen were not arrested at the T1 stage (Fig. 6*D* and Table I). The B cells in Btk^- 3-83$\mu\delta$/Bcl-2 double Tg spleens were largely $IgM^{high}CD21^+CD23^+$, indicating an almost complete block in B cell development from transitional stage T2 into the mature B cell compartment. Compared with their Btk^- 3-83$\mu\delta$ single Tg littermates, a larger fraction of splenic B cells in Btk^- 3-83$\mu\delta$/Bcl-2

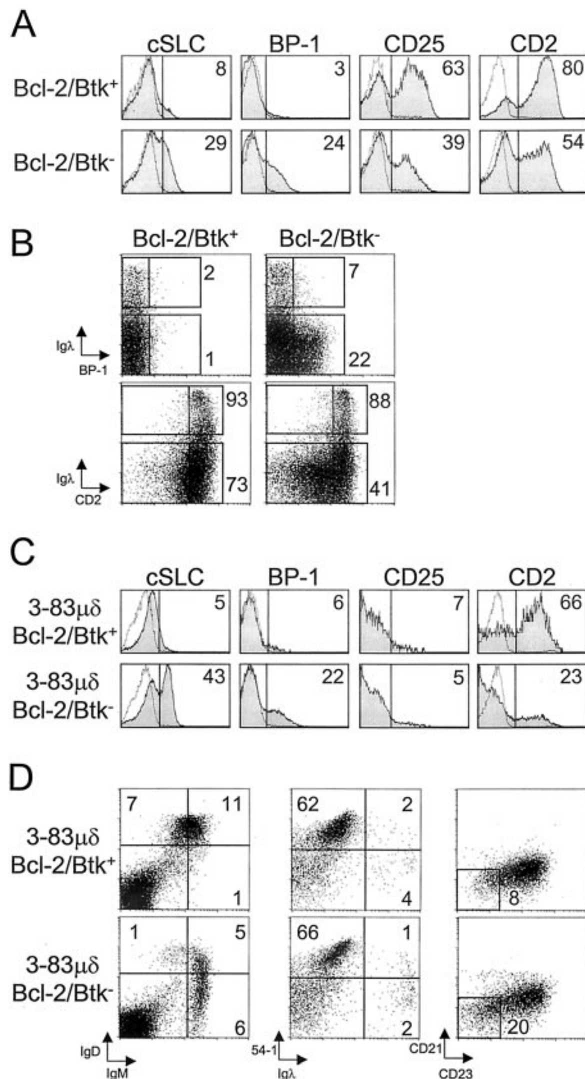

FIGURE 6. Bcl-2 expression does not rescue developmental defects in Btk-deficient non-Tg or 3-83$\mu\delta$ mice. *A,* BM B220^{low}IgM$^-$ pro- and pre-B cells of Btk$^+$ and Btk$^-$ Bcl-2 Tg mice were gated and expression of several developmentally regulated markers is shown as histograms. Numbers indicate percentage of cells positive for the indicated marker and plots are representative for two to four mice of each genotype. *B,* BM B220^{low}IgM$^+$ immature B cells were gated and expression of Igλ and BP-1 or CD2 is shown. Numbers indicate percentage of BP-1- or CD2-positive cells within λ^+ or κ^+ immature B cells. *C,* BM B220^{low} cells of Btk$^+$ and Btk$^-$ 3-83$\mu\delta$/Bcl-2 double Tg mice were gated and analyzed for expression of several developmentally regulated markers as shown in the histograms. *D,* Splenic lymphoid cells were gated and analyzed for IgM/IgD expression and for CD21/CD23 and 54-1/Igλ expression profiles on B220$^+$ gated cells. The percentages of cells within the indicated gates or quadrants are given. Data represent three mice analyzed per genotype.

double Tg mice differentiated into CD21$^+$CD23$^+$ B cells (>80% compared with <50%, respectively; Fig. 6*D*).

From these data, we conclude that an extended time window per cell for pre-B cell differentiation by enforced expression of Bcl-2 was not sufficient to rescue the maturation defects in Btk-deficient pre-B cells. However, the expression of Bcl-2 rescued the peripheral B cell arrest at the T1 stage in Btk$^-$ 3-83$\mu\delta$ mice and consequently B cells were arrested at the T2 stage, as in non-Tg Btk-deficient mice.

Discussion

Btk is essential at two distinct steps in B cell development in the mouse 1) at the developmental progression of CD43$^+$CD2$^-$ large cycling into CD43$^-$CD2$^+$ small resting pre-B cells and 2) at the checkpoint for selection of immature IgM^{high} T2 cells into the pool of long-lived IgM^{low} mature B cells (5, 19, 28, 30, 31, 35, 36). In this report, we show that in Btk-deficient mice premature expression of the 3-83$\mu\delta$ BCR amplifies the cellular maturation defects in immature B cells in the BM and results in an arrest of peripheral B cell development at the transition of T1 into T2 cells in the spleen. Both defects are largely restored when Btk-deficient 3-83$\mu\delta$ mice are crossed onto a centrally deleting background where the B cells were targeted for receptor editing. In contrast, enforced expression of the apoptosis inhibitor Bcl-2, which prolongs (pre-) B cell life span, does not rescue the maturation defects in Btk-deficient 3-83$\mu\delta$ immature B cells in the BM, but does restore the development of T2 B cells in the spleen.

Expression of the pre-BCR triggers IL-7-driven proliferative expansion, allelic exclusion, cellular maturation of pre-B cells, and the initiation of L chain rearrangement (1, 2). We have previously shown that Btk has an important function in the regulation of the substantial changes in cell phenotype that accompanies pre-B cell maturation, including down-regulation of SLC, CD43, and BP-1, and up-regulation of CD2, CD25, and MHC class II (5, 8). However, the functional significance of the modulation of the individual surface markers at the pre-B cell stage is largely unknown, with the exception of SLC. The down-regulation of SLC expression is assumed to limit the capacity of pre-B cells to proliferate. Hence, after several divisions, these cycling cells fall into a resting state where L chain rearrangement is initiated. At this stage of B cell development, Btk and SLP-65 have synergistic roles as tumor suppressors to limit pre-B cell expansion, most likely by signaling the down-regulation of SLC expression (8). Recent analyses of RNA expression profiles identified a major change in gene expression at the transition from cycling into resting pre-B cells, as ~1000 genes were found with differential expression with a magnitude of >2 (37, 38). In flow cytometric analyses, we observed similar differences between Btk-deficient and WT B cells for surface and intracellular expression of CD2, CD25, and MHC class II in cμ^+ pre-B cells (S.M. and R.W.H., unpublished results), indicating that the absence of Btk affects the initiation of the expression of these genes rather than transport of existing protein from cytoplasmic stores to the cell surface. It is therefore possible that Btk is directly or indirectly involved in the regulation of a substantial proportion of the genes required for the initiation of new differentiation programs in resting pre-B cells.

In this context, we found that premature expression of the prerearranged 3-83$\mu\delta$ BCR, which accelerates B cell development (34, 39), is not completely able to replace pre-BCR function, as cellular maturation in these mice was impaired, when compared with non-Tg mice. Nevertheless, the aberrant phenotype of 3-83$\mu\delta$ immature B cells did not seem to significantly affect their capabilities for further maturation in the spleen. In contrast, in the absence of Btk we found a much more defective modulation of marker expression in the BM and a severe decrease in absolute B cell numbers in the spleen, reflecting an almost complete arrest of B cell development at the transition of T1 to T2 immature B cells in the spleen. This contrasts with the arrest in non-Tg Btk-deficient mice, which is at the next developmental transition from T2 into mature B cells.

The cells generated in the BM of the Btk$^-$ 3-83$\mu\delta$ mice were shown to home into the spleen, even in the presence of competitive Btk$^+$ 3-83$\mu\delta$ B cells. In BrdU-labeling studies, we showed that the

numbers of B cells immigrating from the BM in the spleen within 24 h is similar in Btk^+ and Btk^- 3-83$\mu\delta$ mice. However, Btk^- 3-83$\mu\delta$ B cells are efficiently deleted upon arrival in the spleen. It is not likely that the elimination of Btk^- 3-83$\mu\delta$ B cells is due to autoreactivity of the 3-83$\mu\delta$ BCR as 1) the deletion of B cells takes place in the spleen and not in the BM; 2) immature B cells in the BM did not manifest down-regulation of CD19 and IgM surface expression; 3) most splenic B cells express the 3-83$\mu\delta$ Id 54-1, indicating that they did not perform receptor editing; and 4) when the Btk^- 3-83$\mu\delta$ B cells were on a centrally deleting background, the induction of receptor editing even increased survival of T2 peripheral B cells. Instead, our finding that the early T1 to T2 block could be restored by enforced Bcl-2 expression indicates that Btk is required for the survival of T2 cells that carry the 3-83$\mu\delta$ BCR. Interestingly, 3-83$\mu\delta$ Tg and 3-83$\mu\delta$ Bcl-2 double Tg $Lyn^{-/-}$ B cells were also found to be arrested at the T1 and T2 immature B cell stage in the spleen, respectively (40), further supporting synergistic roles for Lyn and Btk in peripheral B cell survival (41). In this respect, the function of CD45 and CD19 in peripheral B cell survival appears to be different, as 3-83$\mu\delta$ Tg $CD45^{-/-}$ and $CD19^{-/-}$ mice showed relatively mild defects in the transitional B cell compartment in the spleen (39, 42, 43).

We have previously shown that lack of Btk did not abolish the receptor editing competence of immature B cells (20). Surprisingly, we found that when Btk-deficient 3-83$\mu\delta$ mice were crossed onto a centrally deleting background and the B cells were targeted for receptor editing, both the cellular maturation defects of immature B cells in the BM and the defective T1 to T2 transition of splenic B cells are restored. It has been shown that B cells with an autoreactive BCR are delayed for 2–12 h in the small pre-B cells compartment where L chain rearrangements take place (17, 18). This delay may provide an extended time window per cell, which could be sufficient to compensate for the impaired cellular maturation of Btk^- pre-B cells. Alternatively, it is also possible that engagement of the BCR with membrane-bound autoantigens induces distinct Btk-independent signaling pathways that drive cellular maturation of editing immature B cells. However, enforced expression of Bcl-2 did not improve cellular maturation of non-Tg or 3-83$\mu\delta$ Btk-deficient immature B cells, indicating that increased life span is not sufficient to correct the Btk-mediated pre-BCR maturation signals. Therefore, our observations would be consistent with the hypothesis that an autoreactive BCR undergoing receptor editing transmits an additional unique signal that enhances cellular maturation of editing pre-B/immature B cells, next to the signals required for the receptor editing events, such as expression of the recombination-activating gene proteins. Such an additional signal would not be provided by an innocuous BCR, as on a nondeleting MHC class I background 3-83$\mu\delta$ immature B cells showed defective cellular maturation. Additional experiments are required to investigate whether BCR stimulated by autoantigens indeed provide maturation signals for editing cells and whether such signals affect the capacity of immature B cells to continue receptor editing or their sensitivity to Ag-induced apoptosis (13).

In this context, the finding that in Btk-deficient mice κ^+ B cells show a more profound cellular maturation defect than λ^+ B cells may suggest that in these mice λ^+ B cells have performed extensive receptor editing. This may be supported by our previous analysis of the kinetics of pre-B cell differentiation in vivo (5, 20). We observed that in Btk-deficient mice, λ^+ B cells manifest a specific developmental delay within the small pre-B cell compartment of ~3 h, accompanied by a decreased production rate. Therefore, the residual λ^+ B cell population in Btk-deficient mice may develop relatively late and consequently contain more cells that have performed receptor editing. Additional experiments are required to directly determine the extent of receptor editing in Btk-deficient mice.

In conclusion, we have shown that in the absence of Btk 3-83$\mu\delta$ B cells are quite efficiently deleted during their development, whether they are on a nondeleting background or on a centrally deleting background. On the nondeleting background, the premature BCR expression amplifies the cellular maturation defects in Btk-deficient immature B cells, finally resulting in defective survival at the T1 to T2 transition in the spleen. On the autoreactive MHC class I background, most immature B cells are deleted in the BM, but cellular maturation defects are significantly reduced and edited B cells that reach the spleen no longer exhibit the T1 to T2 arrest. Our results suggest that at the immature B cell stage in the BM, BCR stimulation by autoantigens does not only induce receptor editing, but also enhances cellular maturation.

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References

1. Melchers, F., E. ten Boekel, T. Seidl, X. C. Kong, T. Yamagami, K. Onishi, T. Shimizu, A. G. Rolink, and J. Andersson. 2000. Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. *Immunol. Rev. 175:33*.
2. Meffre, E., R. Casellas, and M. C. Nussenzweig. 2000. Antibody regulation of B cell development. *Nat. Immunol. 1:379*.
3. Kurosaki, T. 2002. Regulation of B cell fates by BCR signaling components. *Curr. Opin. Immunol. 14:341*.
4. Jumaa, H., B. Wollscheid, M. Mitterer, J. Wienands, M. Reth, and P. J. Nielsen. 1999. Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. *Immunity 11:547*.
5. Middendorp, S., G. M. Dingjan, and R. W. Hendriks. 2002. Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice. *J. Immunol. 168:2695*.
6. Jumaa, H., M. Mitterer, M. Reth, and P. J. Nielsen. 2001. The absence of SLP65 and Btk blocks B cell development at the preB cell receptor-positive stage. *Eur. J. Immunol. 31:2164*.
7. Flemming, A., T. Brummer, M. Reth, and H. Jumaa. 2003. The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. *Nat. Immunol. 4:38*.
8. Kersseboom, R., S. Middendorp, G. M. Dingjan, K. Dahlenborg, M. Reth, H. Jumaa, and R. W. Hendriks. 2003. Bruton's tyrosine kinase cooperates with the B cell linker protein SLP-65 as a tumor suppressor in Pre-B cells. *J. Exp. Med. 198:91*.
9. Hayashi, K., M. Yamamoto, T. Nojima, R. Goitsuka, and D. Kitamura. 2003. Distinct signaling requirements for Dμ selection, IgH allelic exclusion, Pre-B cell transition, and tumor suppression in B cell progenitors. *Immunity 18:825*.
10. Conley, M. E., J. Rohrer, L. Rapalus, E. C. Boylin, and Y. Minegishi. 2000. Defects in early B-cell development: comparing the consequences of abnormalities in pre-BCR signaling in the human and the mouse. *Immunol. Rev. 178:75*.
11. Russell, D. M., Z. Dembic, G. Morahan, J. F. Miller, K. Burki, and D. Nemazee. 1991. Peripheral deletion of self-reactive B cells. *Nature 354:308*.
12. Melamed, D., J. A. Kench, K. Grabstein, A. Rolink, and D. Nemazee. 1997. A functional B cell receptor transgene allows efficient IL-7-independent maturation of B cell precursors. *J. Immunol. 159:1233*.
13. Melamed, D., R. J. Benschop, J. C. Cambier, and D. Nemazee. 1998. Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. *Cell 92:173*.
14. Melamed, D., and D. Nemazee. 1997. Self-antigen does not accelerate immature B cell apoptosis, but stimulates receptor editing as a consequence of developmental arrest. *Proc. Natl. Acad. Sci. USA 94:9267*.
15. Nemazee, D. A., and K. Burki. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature 337:562*.
16. Tiegs, S. L., D. M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med. 177:1009*.
17. Casellas, R., T. A. Shih, M. Kleinewietfeld, J. Rakonjac, D. Nemazee, K. Rajewsky, and M. C. Nussenzweig. 2001. Contribution of receptor editing to the antibody repertoire. *Science 291:1541*.
18. Oberdoerffer, P., T. I. Novobrantseva, and K. Rajewsky. 2003. Expression of a targeted λ1 light chain gene is developmentally regulated and independent of Igκ rearrangements. *J. Exp. Med. 197:1165*.
19. Hendriks, R. W., M. F. de Bruijn, A. Maas, G. M. Dingjan, A. Karis, and F. Grosveld. 1996. Inactivation of Btk by insertion of *lacZ* reveals defects in B cell development only past the pre-B cell stage. *EMBO J. 15:4862*.
20. Dingjan, G. M., S. Middendorp, K. Dahlenborg, A. Maas, F. Grosveld, and R. W. Hendriks. 2001. Bruton's tyrosine kinase regulates the activation of gene

rearrangements at the λ light chain locus in precursor B cells in the mouse. *J. Exp. Med. 193:1169*.

21. Dingjan, G. M., A. Maas, M. C. Nawijn, L. Smit, J. S. Voerman, F. Grosveld, and R. W. Hendriks. 1998. Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase. *EMBO J. 17:5309*.
22. Karasuyama, H., A. Rolink, Y. Shinkai, F. Young, F. W. Alt, and F. Melchers. 1994. The expression of Vpre-B/λ5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. *Cell 77:133*.
23. Rolink, A. G., J. Andersson, and F. Melchers. 1998. Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. *Eur. J. Immunol. 28:3738*.
24. Petrenko, O., A. Beavis, M. Klaine, R. Kittappa, I. Godin, and I. R. Lemischka. 1999. The molecular characterization of the fetal stem cell marker AA4. *Immunity 10:691*.
25. Arakawa, H., T. Shimizu, and S. Takeda. 1996. Re-evaluation of the probabilities for productive arrangements on the κ and λ loci. *Int. Immunol. 8:91*.
26. Engel, H., A. Rolink, and S. Weiss. 1999. B cells are programmed to activate κ and λ for rearrangement at consecutive developmental stages. *Eur. J. Immunol. 29:2167*.
27. Wicker, L. S., and I. Scher. 1986. X-linked immune deficiency (*xid*) of CBA/N mice. *Curr. Top. Microbiol. Immunol. 124:87*.
28. Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A. B. Kantor, L. A. Herzenberg, et al. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity 3:283*.
29. Hertz, M., and D. Nemazee. 1997. BCR ligation induces receptor editing in $IgM^{+}IgD^{-}$ bone marrow B cells in vitro. *Immunity 6:429*.
30. Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 1999. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J. Exp. Med. 190:75*.
31. Allman, D., R. C. Lindsley, W. DeMuth, K. Rudd, S. A. Shinton, and R. R. Hardy. 2001. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J. Immunol. 167:6834*.
32. Opstelten, D., and D. G. Osmond. 1983. Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic μ-chain-bearing cells in normal mice. *J. Immunol. 131:2635*.
33. Rolink, A., U. Grawunder, D. Haasner, A. Strasser, and F. Melchers. 1993. Immature surface Ig^{+} B cells can continue to rearrange κ and λ L chain gene loci. *J. Exp. Med. 178:1263*.
34. Lang, J., B. Arnold, G. Hammerling, A. W. Harris, S. Korsmeyer, D. Russell, A. Strasser, and D. Nemazee. 1997. Enforced Bcl-2 expression inhibits antigen-mediated clonal elimination of peripheral B cells in an antigen dose-dependent manner and promotes receptor editing in autoreactive, immature B cells. *J. Exp. Med. 186:1513*.
35. Scher, I. 1982. CBA/N immune defective mice; evidence for the failure of a B cell subpopulation to be expressed. *Immunol. Rev. 64:117*.
36. Su, T. T., and D. J. Rawlings. 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *J. Immunol. 168:2101*.
37. Hoffmann, R., L. Bruno, T. Seidl, A. Rolink, and F. Melchers. 2003. Rules for gene usage inferred from a comparison of large-scale gene expression profiles of T and B lymphocyte development. *J. Immunol. 170:1339*.
38. Hoffmann, R., and F. Melchers. 2003. A genomic view of lymphocyte development. *Curr. Opin. Immunol. 15:239*.
39. Shivtiel, S., N. Leider, O. Sadeh, Z. Kraiem, and D. Melamed. 2002. Impaired light chain allelic exclusion and lack of positive selection in immature B cells expressing incompetent receptor deficient of CD19. *J. Immunol. 168:5596*.
40. Meade, J., C. Fernandez, and M. Turner. 2002. The tyrosine kinase Lyn is required for B cell development beyond the T1 stage in the spleen: rescue by over-expression of Bcl-2. *Eur. J. Immunol. 32:1029*.
41. Satterthwaite, A. B., C. A. Lowell, W. N. Khan, P. Sideras, F. W. Alt, and O. N. Witte. 1998. Independent and opposing roles for Btk and lyn in B and myeloid signaling pathways. *J. Exp. Med. 188:833*.
42. Buhl, A. M., D. Nemazee, J. C. Cambier, R. Rickert, and M. Hertz. 2000. B-cell antigen receptor competence regulates B-lymphocyte selection and survival. *Immunol. Rev. 176:141*.
43. Shivtiel, S., N. Leider, and D. Melamed. 2002. Receptor editing in CD45-deficient immature B cells. *Eur. J. Immunol. 32:2264*.