Impaired Precursor B Cell Differentiation in Bruton's Tyrosine Kinase-Deficient Mice[1]

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Bruton's tyrosine kinase (Btk) is a cytoplasmic signaling molecule that is crucial for precursor (pre-B) cell differentiation in humans. In this study, we show that during the transition of large cycling to small resting pre-B cells in the mouse, Btk-deficient cells failed to efficiently modulate the expression of CD43, surrogate L chain, CD2, and CD25. In an analysis of the kinetics of pre-B cell differentiation in vivo, Btk-deficient cells manifested a specific developmental delay within the small pre-B cell compartment of ~3 h, when compared with wild-type cells. Likewise, in in vitro bone marrow cultures, Btk-deficient large cycling pre-B cells showed increased IL-7 mediated expansion and reduced developmental progression into noncycling $CD2^+CD25^+$ surrogate L chain-negative small pre-B cells and subsequently into Ig-positive B cells. Furthermore, the absence of Btk resulted in increased proliferative responses to IL-7 in recombination-activating gene-1-deficient pro-B cells. These findings identify a novel role for Btk in the regulation of the differentiation stage-specific modulation of IL-7 responsiveness in pro-B and pre-B cells. Moreover, our results show that Btk is critical for an efficient transit through the small pre-B cell compartment, thereby regulating cell surface phenotype changes during the developmental progression of cytoplasmic μ H chain expressing pre-B cells into immature IgM^+ B cells. ***The Journal of Immunology,*** **2002, 168: 2695–2703.**

Bruton's tyrosine kinase (Btk)[3] belongs to the Tec family of cytoplasmic protein tyrosine kinases and plays an essential role in B lymphocyte development and function (1, 2). In mature B cells, Btk is tyrosine phosphorylated and its kinase activity is increased upon B cell receptor (BCR) stimulation (3–5).

Mutations in the *Btk* gene lead to X-linked agammaglobulinaemia (XLA) in humans and X-linked immunodeficiency (*xid*) in the mouse (6–9). XLA is characterized by recurrent bacterial infections, and very low serum Ig levels of all classes due to the lack of plasma cells in the secondary lymphoid organs. There is a severe deficiency of peripheral B cells, and those B cells that are present have an aberrant IgM^{high} phenotype (10). In the bone marrow (BM) of XLA patients, the numbers of cytoplasmic μ H chain (cμ)-expressing pre-B cells are variable, but in most cases reduced; those pre-B cells present are mainly nonproliferating small cells (11–13). The absence of Btk apparently results in deficient expansion of the earliest cμ-expressing pre-B cells. Therefore, the XLA disease phenotype most likely reflects defective signaling through the pre-BCR, by which the expression of a functional μ H chain normally is monitored, together with surrogate L chain (SLC; Ref. 14). As most patients have substantial numbers of pro-B cells in their BM, XLA generally results in an increased ratio of pro-B to pre-B cells (11–13).

In contrast, Btk deficiency in the mouse is associated with an impairment of peripheral B cell maturation, without a major early B cell developmental block (15–17). In *xid* mice the mature $IgM^{low}IgD^{high}$ B cell populations in spleen and lymph nodes are severely reduced, B-1 B cells are absent, and serum IgM and IgG3 levels are low. *Xid* B cells do not proliferate upon surface IgM (sIgM) stimulation. The most obvious difference between the BM B cell compartment of wild-type (WT) mice and Btk-deficient mice is the reduction in mature recirculating cells (15–17). The absolute numbers of pre-B cells that are generated in the BM of Btk-deficient mice are normal, and Btk-deficient B cell precursors in the BM have the same kinetics of turnover (18, 19). Nevertheless, several lines of evidence point at a role for Btk at the transition of pre-B cells into immature surface Ig^+ B cells, thereby indicating the involvement of Btk in pre-BCR signaling in the mouse. Using an in vivo competition assay in heterozygous $Btk^{+/-}$ female mice, we identified a small but significant selective disadvantage of Btk-deficient cells to contribute to the IgM^+IgD^- immature B cells stage in the BM (17). Our recent finding of an intrinsic reduction of Ig λ L chain usage in Btk-deficient B cells in the mouse implicated Btk in the activation of gene rearrangements at the λ L chain locus (20). Furthermore, while mice deficient for another Tec kinase family member, Tec, showed normal B cell development, an almost complete block at the $CD43^+B220^+$ stage of B cell development was observed in Btk/Tec double-deficient mice (21). These results indicated that Btk is critically involved in pre-BCR-mediated signaling in the mouse and that Tec is able to compensate for the loss of Btk during early B cell development. A role for Btk in pre-BCR signaling would be consistent with the recent finding that Btk is able to function in CD79b (Igβ)-mediated signaling in recombination-activating gene (RAG)-2-deficient pro-B cells (22).

To examine the role of Btk at the pre-BCR checkpoint in vivo, we compared the expression of early B cell surface markers in the BM from Btk-deficient and WT control mice. In this report, we show that Btk is involved in the pre-BCR-dependent induction of

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[3] Abbreviations used in this paper: Btk, Bruton's tyrosine kinase; BCR, B cell receptor; BM, bone marrow; FSC, forward scatter; WT, wild type; *xid*, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia; SLC, surrogate L chain; RAG, recombination-activating gene; sIgM, surface IgM; BrdU, 5-bromo-2′deoxyuridine; cμ, cytoplasmic μ H chain; 7-AAD, 7-aminoactinomycin-D.

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cell surface phenotype changes during the progression of large cycling $c\mu^+$ pre-B cells into surface Ig^+ immature B cells, thereby affecting the transit time through the small pre-B cell compartment. In addition, we performed IL-7-driven BM cultures, which point at a role for Btk in the differentiation stage-specific modulation of IL-7 responsiveness in early murine B cell development, even in stages preceding $c\mu$ expression.

Materials and Methods

Mice and genotyping

Btk$^-$/*lacZ* mice (17) were crossed onto a C57BL/6 background for >6 generations. Specific experiments were additionally performed with Btk mice on a mixed C57BL/6 × 129 or C57BL/6 × FVB background, but no significant influence of the genetic background was observed. *RAG-1*$^{-/-}$ mice (23) were on a 129/Sv background. All mice were bred and maintained in the animal care facility at the Erasmus Medical Center, Rotterdam (Rotterdam, The Netherlands). To determine the Btk genotype, tail DNA was analyzed by Southern blotting of *Bam*HI digests, as described (17). The *RAG-1* genotype was determined using a 1.9-kb partial *RAG-1* probe (encoding amino acid positions 387-1008, kindly provided by D. van Gent, Erasmus Medical Center) to hybridize to *Bam*HI-*Nco*I genomic digests (23).

Flow cytometric analysis

Preparations of single-cell suspensions, flow cytometry, and determination of β-galactosidase activity by loading cells with fluorescein-di-β-D-galactopyranoside substrate (Molecular Probes Europe, Leiden, The Netherlands), have previously been described (17, 24). Events ($1-3 \times 10^5$) were scored using a FACSCalibur flow cytometer and analyzed by CellQuest software (BD Biosciences, Mountain View, CA). The following mAbs were obtained from BD PharMingen (San Diego, CA): FITC-conjugated anti-BP-1/6C3, anti-B220/RA3-6B2, anti-κ(R5-240), and anti-IgM (II/41); PE-conjugated anti-CD2, anti-κ(187.1), anti-CD25, and anti-CD43; Cy-Chrome-conjugated anti-B220/RA3-6B2 and biotinylated anti-IL7R, anti-κ(187.1), anti-λ_1/λ_2(R26-46), and anti-IgM (II/41). PE-conjugated IgD was obtained from Southern Biotechnology Associates (Birmingham, AL). Biotinylated anti-SLC Ab LM34 (25) was kindly provided by A. Rolink (Basel Institute for Immunology, Basel, Switzerland). Secondary Abs were PE-, tricolor-, or APC-conjugated streptavidin, purchased from Caltag Laboratories (Burlingame, CA).

For intracellular flow cytometric detection of cytoplasmic Ig H or L chain or SLC, the following Abs were applied: FITC-conjugated anti-Igκ (R5-240, BD PharMingen) or polyclonal anti-Ig μ H chain (Jackson Immunoresearch Laboratories, West Grove, PA) and biotinylated anti-λ_1/λ_2 (BD PharMingen), or anti-SLC (LM34). Cells were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin.

Cell cycle analysis

To analyze cell cycle status of cultured cell suspensions, cells were incubated in ice-cold ethanol for >2 h. Subsequently, cells were incubated at room temperature for 30 min in PBS containing 0.02 mg/ml propidium iodide, 0.1% v/v Triton X-100, and 0.2 mg/ml RNase, left overnight at 4°C, and analyzed with a FACSCalibur flow cytometer. Doublet cells were excluded from the analysis by measuring peak area and width. For simultaneous analysis of cell surface markers, $c\mu$ expression, and DNA content, we used a method described by Constantinescu and Schlissel (26). Cells were sequentially stained for surface CD2 expression using PE-labeled anti-CD2, for $c\mu$ using polyclonal anti-μ H chain, and 7-aminoactinomycin-D (7-AAD, Molecular Probes Europe).

IL-7-driven BM cultures

Primary pre-B cell cultures were essentially performed as described previously (20), using erythrocyte-depleted total BM cell suspensions. For specific experiments, BM cell suspensions were depleted of $sIgM^+$ cells, using biotinylated anti-IgM and streptavidin-coated microbeads, as described (20). Recombinant murine IL-7 was from R&D Systems (Minneapolis, MN). To measure IL-7-dependent proliferative responses, cells were cultured in flat-bottom 96-well plates for 5 days with various amounts of IL-7 at different cell concentrations ($0.25–1 \times 10^5$ cells/well). Cultures were pulsed for 24 h with 0.5 μCi/well of [^{3}H]thymidine, harvested on glass-fiber filters, and the incorporated radioactivity was determined using a beta counter, according to standard procedures.

In vivo 5-bromo-2′-deoxyuridine (BrdU) labeling

BrdU (Sigma Aldrich, St. Louis, MO) was dissolved in PBS at 2 mg/ml. Mice were injected i.p. with 200 μl, and sacrificed at various time points. Total BM cell suspensions were analyzed by flow cytometry for BrdU incorporation, using the BrdU flow kit (BD PharMingen) in conjunction with cell surface marker expression.

Results

Btk-cμ^+ pre-B cells show a defective surface marker expression profile

To investigate the involvement of Btk in pre-BCR signaling, we compared the pre-B cell compartment in the BM of Btk^+ and Btk^- mice. Consistent with published findings (16, 17, 21), we did not observe a major early B cell developmental block in Btk^- mice, but only a small increase in the fraction of $CD43^+$ cells within the population of $sIgM^-B220^+$ pro-/pre-B cells: ~20–25% in Btk^+ and ~50–55% in Btk^- mice (Fig. 1*A*).

About one-fifth of all pre-B cells have been reported to express μ H chain and L chains in their cytoplasm, without depositing IgM on the cell surface (27). These cells may have down-regulated sIgM expression due to autoantigen binding, or they may produce Ig H and L chains that do not pair (14, 27). As previously described (20), surface Ig^- B cell precursors of Btk^- mice showed reduced expression of cytoplasmic L chain. Surface Ig^- B cells (16 ± 3%) expressed κ L chain in the cytoplasm, compared with 23 ± 2% in Btk^+ mice, while the frequency of cytoplasmic λ expression pre-B cells was reduced by a factor ~3 (Fig. 1*B*). These findings suggest reduced or delayed Ig L chain rearrangement.

Signaling through the pre-BCR initiates proliferative expansion of those cells with a productively rearranged Ig H chain. Subsequently, these cells progress into small resting pre-B cells that have changed their cell surface phenotype and initiate κ and λ L chain rearrangement (14). Therefore, we analyzed IgM^-B220^+ B cell precursors for the expression of several cell surface markers in four-color flow cytometric experiments (Fig. 1*C*). In Btk^- mice, the IgM^-B220^+ compartment contained a unique subpopulation of $CD43^{low}$ cells which manifested increased expression of the BP-1 molecule, a metallopeptidase that is selectively induced coincident with IL-7-driven pre-B cell proliferation (28). As soon as a pre-BCR is expressed, the synthesis of SLC is turned off (29). However, Btk^- cells had an increase in the fraction of IgM^-B220^+ cells that expressed SLC in their cytoplasm, as compared with Btk^+ cells (Fig. 1*C*). In a specific analysis of $c\mu^+$ pre-B cells, Btk^- mice showed significantly reduced expression of the IL-2R CD25 (30 ± 6%), and the CD2 adhesion molecule (32 ± 4%), as compared with Btk^+ mice (66 ± 7% and 70 ± 6%, respectively). These molecules were previously reported to be up-regulated upon pre-BCR signaling (30, 31).

Collectively, these findings indicated that the absence of Btk resulted in a defective progression of $SLC^+CD43^+CD2^-CD25^-$ into $SLC^-CD43^-CD2^+CD25^+c\mu^+$ pre-B cells. Analysis of the absolute numbers of the various B-lineage subpopulations in the BM (Table I) revealed that in Btk^- mice the size of the total population of $CD2^-$ $c\mu^+$ early pre-B cells was increased with a factor of ~2.4, whereas the total population of $CD2^+$ $c\mu^+$ late pre-B cells was decreased with a similar factor.

The developmental progression to $CD2^+$ late pre-B cells normally correlates with a change in cell cycle status, from large cycling into small resting pre-B cells (26). This was confirmed by the finding in Btk^+ mice of ~45% of $CD2^-$ and ~10% of $CD2^+c\mu^+$ pre-B cells in S, M, or G_2 phase, using 7-AAD staining for DNA content (Fig. 1*C*). In contrast, the $CD2^-c\mu^+$ pre-B cell population in Btk^- mice contained only ~23% of cycling cells,

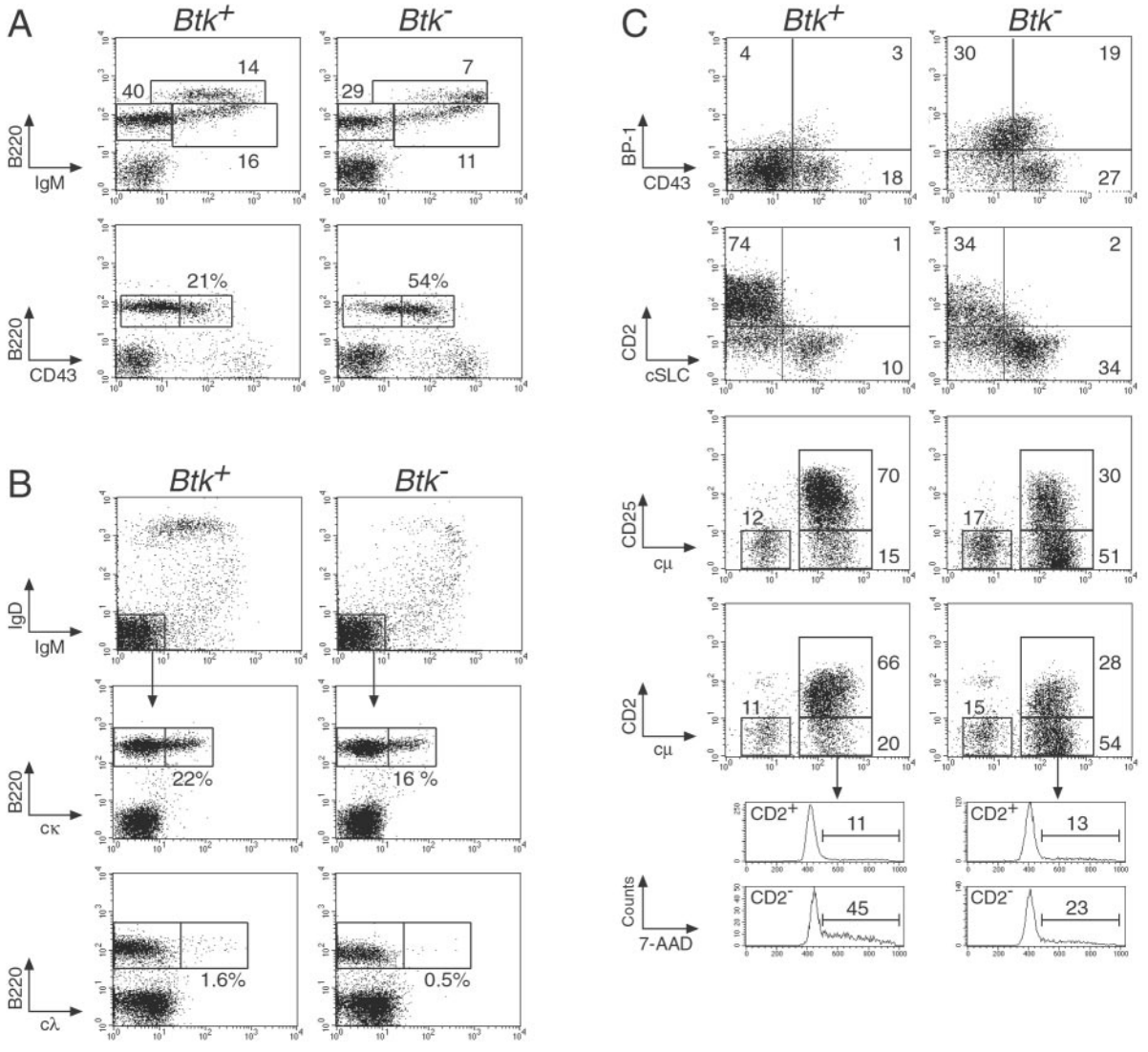

FIGURE 1. Characterization of the pre-B cell compartment in Btk$^+$ and Btk$^-$ mice. *A*, BM cell suspensions were stained with mAbs to B220, CD43, and IgM. The expression profiles of B220 and sIgM are shown (*top*). sIgM$^-$ cells were analyzed for B220 and CD43 (*bottom*). Percentages of CD43$^+$ cells within the indicated B220$^+$ gate are given. *B*, BM cell suspensions were stained with mAbs to detect surface expression of IgM, IgD, B220, and cytoplasmic (c) expression of κ or λ L chain. The percentages of cκ^+ and cλ^+ cells within the IgM$^-$IgD$^-$ B220$^+$ subpopulation are given. *C*, Four-color flow cytometric analyses of BM cell suspensions, using mAbs to B220 and IgM, together with various additional mAbs. B220$^+$IgM$^-$ pro-B/pre-B cells were electronically gated and analyzed for the indicated markers. Cell cycle status was determined in cμ^+CD2$^+$ and cμ^+CD2$^-$ cells, using 7-AAD. The percentages of cycling cells (S, M, or G_2 phase) are indicated. Data shown are representative of three to eight mice examined.

i.e., about half the number found in Btk$^+$ mice. However, as the size of the total population of CD2$^-$cμ^+ pre-B cells was increased with a factor ~2.4 in Btk$^-$ mice, we conclude that Btk$^+$ and Btk$^-$ mice contain comparable numbers of large cycling cμ^+ cells (~0.8×10^6 cells per hind leg).

In summary, the absence of Btk in pre-BCR signaling resulted in defective down-regulation of CD43 and SLC, defective up-regulation of CD2 and CD25, augmented expression of BP-1, whereas in vivo proliferative expansion of pre-B cells appeared to be unaffected.

Table I. *B-lineage subpopulations in Btk$^+$ and Btk$^-$ mice*

Cell Population	Btk$^+$ (n = 7)	Btk$^-$ (n = 7)	p Values
Total B220$^+$ cells	13.8 ± 2.19[a]	11.1 ± 2.78	NS
Total sIgM$^-$B220$^+$ cells	8.70 ± 1.58	7.63 ± 2.35	NS
Pro-B (cμ^-)	1.16 ± 0.36	1.24 ± 0.50	NS
Early pre-B (cμ^+CD2$^-$)	1.56 ± 0.75	3.82 ± 1.41	0.0028
Late pre-B (cμ^+CD2$^+$)	5.63 ± 1.29	2.32 ± 0.72	<0.0001
Immature B cells (sIgM$^+$sIgD$^-$)	2.22 ± 0.51	2.01 ± 0.57	NS
Mature B cells (sIgM$^+$sIgD$^-$)	1.83 ± 0.54	0.86 ± 0.37	0.0022

[a] Absolute number of cells (mean ± SD × 10^6) per hind leg.

Btk⁻ cells show a developmental delay within the small pre-B cell compartment in vivo

The inefficient induction of cell surface phenotype changes in Btk⁻ mice at the pre-B cell stage was still noticeable at the next developmental stage of IgM⁺ immature B cells. As shown by flow cytometric analysis (Fig. 2*A*), IgM⁺B220^{low} immature B cells in Btk⁻ mice manifested lower expression of CD2 (66 ± 5%, $n = 9$) and CD25 (19 ± 1%, $n = 12$), compared with immature B cells in Btk⁺ animals (CD2⁺: 90 ± 3%, $n = 8$, and CD25⁺: 36 ± 1%, $n = 12$). In addition, considerable fractions of Btk⁻ immature B cells expressed surface BP-1 (45 ± 3%, $n = 6$) or cytoplasmic SLC (13 ± 3%, $n = 8$), which were not detected in Btk⁺ immature B cells (<3%; Fig. 2*A*).

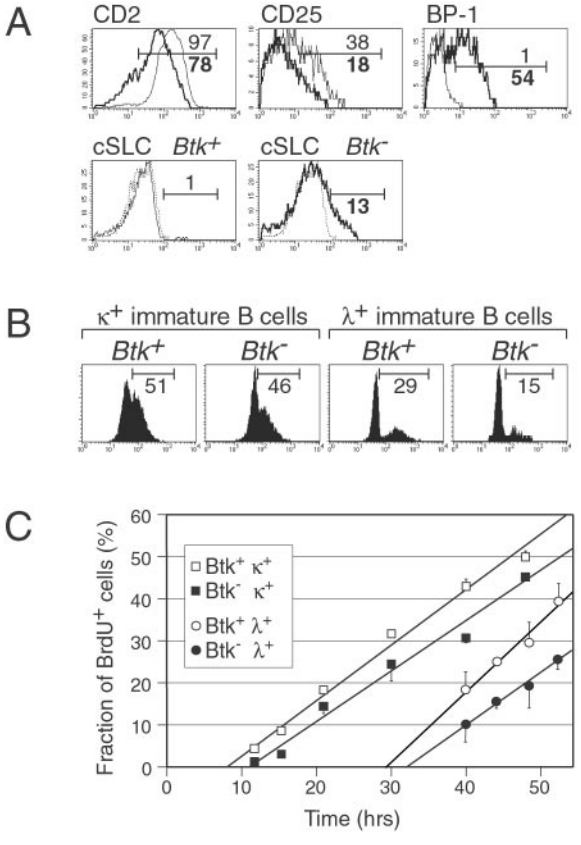

FIGURE 2. Absence of Btk results in delayed developmental progression into immature B cells. *A*, Expression of CD2, CD25, cytoplasmic SLC (cSLC), and BP-1 on B220^{low}IgM⁺ cells in BM of Btk⁺ (*thin lines*) and Btk⁻ mice (*bold lines*). For cSLC analysis, histogram overlays of gated B220⁻ cells are shown as negative control stainings (*dotted lines*). The percentages of positive cells are indicated above the marker line (Btk⁺) and below the marker line in bold (Btk⁻). *B*, BrdU incorporation 48 h after BrdU injection in Btk⁺ and Btk⁻ mice. BM cells were stained for surface expression of B220, IgD, and κ or λ, in conjunction with BrdU. Histograms show BrdU staining in B220⁺IgD⁻ immature B cells, with the percentages of BrdU⁺ cells. *C*, Linear regression analysis of the appearance of BrdU⁺ κ⁺ and λ⁺ immature B cells over time in Btk⁺ (open symbols) and Btk⁻ mice (filled symbols). Each point represents BrdU staining of B220⁺IgD⁻κ⁺/λ⁺ BM cells from three to six mice per group (except for Btk⁻ mice, 30 h: $n = 2$). Entry points and slope values were 8 h and 1.3 (κ, Btk⁺), 11 h and 1.2 (κ, Btk⁻), 29 h and 1.7 (λ, Btk⁺), and 32.5 h and 1.3 (λ, Btk⁻), respectively. As evaluated by Student's *t* test, the fractions of BrdU⁺ cells were significantly lower in κ⁺ Btk⁻ cells as compared with Btk⁺ cells, with *p* values of <0.01 (12, 16, and 40 h), <0.02 (48 h), and 0.05 (21 h). For the 30-h time point, the number of measurements was insufficient for a statistical evaluation. For λ⁺ B cells, the *p* values ranged from <0.0001 to <0.01 for the four time points shown.

To investigate whether the inefficient induction of cell surface phenotype changes in Btk⁻ pre-B and immature B cells would be associated with a delayed developmental progression into IgM⁺ immature B cells, we estimated the transit time through the small pre-B cell compartment. Btk⁺ and Btk⁻ mice were injected with a single dose of the BrdU thymidine analog, which is specifically incorporated into the DNA of large cycling pre-B cells (32–34). BM cells were stained with an anti-BrdU Ab, in conjunction with cell surface analysis for B220, IgD, and κ or λ L chain, and analyzed by flow cytometry. Twelve hours after injection, ~30–35% of Ig⁻ B cell precursors incorporated BrdU, both in Btk⁺ and Btk⁻ mice. At various time points after BrdU injection, we analyzed BrdU incorporation within κ⁺ and λ⁺ IgD⁻ immature B cells. As shown for the 48-h time point in Fig. 2*B*, the fractions of BrdU⁺ cells within the surface κ⁺ or λ⁺ immature B cells were decreased in Btk⁻ mice when compared with Btk⁺ mice. As small pre-B cells are noncycling cells, the first appearance of BrdU⁺ cells within the surface Ig⁺ immature B cells reflects the minimum transit time through the small pre-B compartment. When for κ⁺ immature B cells, the data from six time points were subjected to linear regression analysis (33), it was found that BrdU⁺κ⁺ immature B cells emerged ~8 h after BrdU injection in Btk⁺ mice and only after ~11 h in Btk⁻ mice (Fig. 2*C*). In agreement with reported experiments (33, 34), BrdU⁺λ⁺ immature B cells only emerged ~30 h after BrdU injection. Likewise, linear regression analysis revealed a delay of ~3.5 h for Btk-deficient λ⁺ immature B cells (~32.5 h), as compared with Btk⁺ cells (~29 h).

In summary, we conclude that in Btk⁻ mice the inefficient induction of cell surface phenotype changes in μ⁺ pre-B cells was accompanied by a specific developmental delay within the small pre-B cell compartment of ~3 h. In addition, the immature B cells generated manifest an aberrant surface phenotype.

Btk⁻ pre-B cells show deficient developmental progression in vitro

To further investigate the role of Btk in the developmental progression of cμ⁺ pre-B cells and the initiation of Ig L chain expression, IL-7-driven BM culture experiments were performed, as described (35, 36). Pre-B cells that have a productive Ig H chain rearrangement undergo rapid cell division in response to IL-7. When IgM⁻ BM cell suspensions from Btk⁺ mice were cultured in the presence of 100 U/ml IL-7 for 5 days, the majority of cells consisted of B220⁺IgM⁻ cells, while a significant fraction matured to sIgM⁺IgD⁻ (~10%) or IgM⁺IgD⁺ B cell stages (~5%) (Fig. 3). This differentiation into IgM⁺ cells did not result from an IL-7 insufficiency in culture, as it was also present when higher IL-7 concentrations were used. In contrast, only <5% and <0.5% of the Btk⁻ cells exhibited an IgM⁺IgD⁻ or IgM⁺IgD⁺ profile in culture, respectively. Therefore, these findings indicated that Btk signaling supported the progression from pre-B cell to surface Ig⁺ B cell in vitro in the presence of IL-7. Similar differences were observed, when cells were subsequently cultured for 48 h on S17 stroma cells in the presence of IL-7: ~30% of Btk⁺B220⁺ cells were surface Ig⁺, but only ~5% of Btk⁻B220⁺ cells (Fig. 3). After removal of IL-7, which strongly induces the cells to exit from cell cycle and to further differentiate (36), significant numbers of sIgM⁺ B cells were generated in the Btk⁻ BM cultures. Nevertheless, when compared with Btk⁺ B cells, the maturation of Btk⁻ B cells to the IgM⁺IgD⁺ stage was reduced (Fig. 3). Therefore, the analyses of IL-7-driven BM cultures showed that Btk signaling promotes the maturation of pre-B cells into surface Ig⁺ B cells in the presence of IL-7 in vitro.

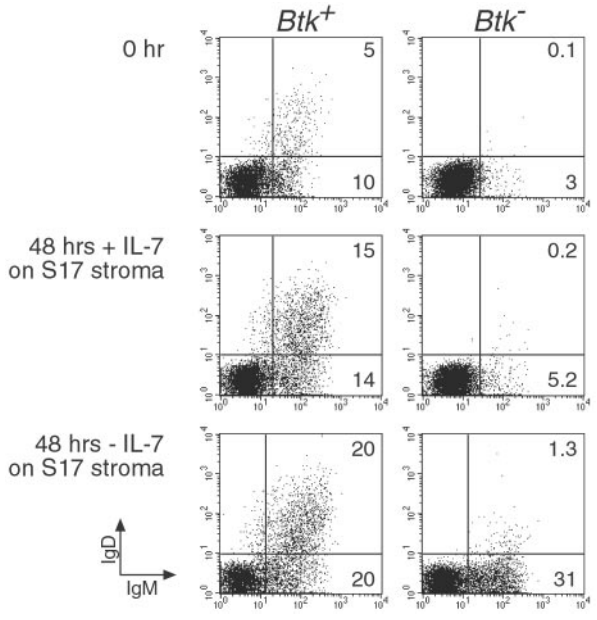

FIGURE 3. Developmental progression of Btk$^+$ and Btk$^-$ cells in IL-7-driven BM cultures. BM cells were cultured in the presence of IL-7 for 5 days and subsequently analyzed ("0 h") or recultured on S17 stroma cells in the presence or absence of IL-7 for 48 h. Cultured cells were stained for B220, IgM, and IgD. B220$^+$ cells were gated and analyzed for the expression of IgM and IgD. Data shown are representative of 10 mice examined in each group.

Btk$^-$ pre-B cells show enhanced IL-7-driven expansion in vitro

Next, we analyzed the kinetics of in vitro B cell development in IL-7-driven BM cultures, using total BM cells from Btk$^+$, Btk$^-$, and Btk$^{+/-}$ mice, without S17 stroma. Similar results were obtained in IL-7-driven BM cultures in the presence of S17 stromal cells, either throughout the 7-day culture period or from day 5 onwards (data not shown).

In these experiments, we used biotinylated Abs to detect sIgM expression, which resulted in higher fluorescence intensity values (up to 2×10^3), as compared with the experiments described in Fig. 3 (up to 5×10^2). We observed that most Btk$^-$B220$^+$ cells stained with the anti-μ H chain Ab after culture with IL-7 (fluorescence intensities ranging from 20 to 100; Fig. 4*A*). These cells were cμ positive, surface, or cytoplasmic κ or λ negative, and expressed SLC both in their cytoplasma and on the cell surface (see *Results*; Fig. 5). Therefore, we concluded that these cells were pre-B cells, and that the anti-μ H chain Ab staining in Btk$^-$ cells reflected high surface pre-BCR expression. In contrast, the Btk$^+$ cells with anti-IgM fluorescence intensities ranging from 20 to 100 were surface κ^+ cytoplasmic SLC$^-$ immature B cells (see *Results*; Fig. 5).

When compared with Btk$^+$ cultures, Btk$^-$ and Btk$^{+/-}$ cultures were characterized by a more rapid loss of mature surface Ig$^+$ cells (Fig. 4*A*, day 3), and a reduced appearance of newly formed Ig$^+$ B cells (Fig. 4*A*, days 5 and 7). From the total cell counts and the flow cytometric analyses of the cells, the expansion rates of sIgM$^-$ and sIgM$^+$B220$^+$ cells during culture were calculated. As shown in Fig. 4*B*, pre-B cell expansion was significantly higher in the cultures from Btk$^-$ mice ($\sim$30$\times$) when compared with Btk$^+$ mice ($<$10$\times$). Only for Btk$^+$ BM cells, the further differentiation of pre-B cells generated in the cultures in the presence of IL-7 resulted in an expansion of mature sIgM$^+$ B cells ($\sim$4$\times$).

Increased proliferation of Btk$^-$ cells was confirmed by analysis of cell cycle and cell size (Fig. 4*C*). After 5 days of culture, 53 $\pm$ 3% of the Btk$^-$ cells ($n = 6$) and only 25 $\pm$ 3% of the Btk$^+$ ($n = 6$) were in the S, M, or G_2 phase of the cell cycle. In these experiments, the Btk$^-$ BM cultures contained higher proportions (77 $\pm$ 6%) of large cells with high forward scatter (FSC) values than the Btk$^+$ BM cultures (42 $\pm$ 7%). To further quantify cell proliferation in the presence of various concentrations of IL-7, we performed [^{3}H]thymidine incorporation experiments. After 5 days of culture, Btk$^-$ cells showed significantly higher proliferative responses to IL-7 when compared with Btk$^+$ cells (Fig. 4*D*). These findings in Btk$^+$ and Btk$^-$ BM cultures demonstrated that in the absence of Btk, IL-7-driven pre-B cell proliferation in vitro is increased.

Enhanced IL-7-driven expansion and decreased developmental progression is intrinsic to Btk$^-$ pre-B cells

The observed differences in IL-7-driven proliferation between Btk$^+$ and Btk$^-$ pre-B cells cannot easily be explained by increased surface expression of IL-7R on these cells in Btk$^-$ mice. In flow cytometric experiments, the proportions of IL-7Rα chain positive cells within the large pre-B cell population in Btk$^+$ and Btk$^-$ mice were 77 $\pm$ 4% and 87 $\pm$ 2%, respectively, while the mean fluorescence intensities of IL-7Rα^+ cells were comparable.

To answer the question of whether the phenotype of Btk$^-$ cells in IL-7-driven BM cultures is an intrinsic feature of Btk$^-$ pre-B cells, or alternatively, originates from a defective BM microenvironment in Btk$^-$ mice, we also investigated BM cultures from Btk$^{+/-}$ mice. Due to random X chromosome inactivation, the pre-B cell compartment in these mice consists of two functionally different populations, i.e., Btk$^+$ and Btk$^-$ (17, 37, 38). In Btk$^{+/-}$ heterozygous mice, IL-7-driven pre-B cell proliferation was intermediate between Btk$^+$ and Btk$^-$ mice, as measured by pre-B cell expansion, cell cycle status, and [^{3}H]thymidine incorporation (Fig. 4, *B–D*). At days 5 and 7, the surface Ig expression profiles and the FSC values in Btk$^{+/-}$ cultures were similar to those of Btk$^-$ cells (Fig. 4, *A* and *C*), indicating a proliferative advantage of Btk$^-$ over Btk$^+$ cells. The presence of a *lacZ* reporter in the *Btk$^-$* allele enabled a separate evaluation of the expansion of Btk$^+$/*lacZ*$^-$ and Btk$^-$/*lacZ*$^+$ cells in Btk$^{+/-}$ cultures. At days 5 and 7, the proportions of *lacZ*$^+$ cells in Btk$^{+/-}$ and Btk$^-$ cultures were comparable, indicating an intrinsic selective advantage of Btk-deficient cells in culture. In these cultures, the majority of IgM$^+$ cells were Btk$^+$/*lacZ*$^-$. Thus, the increased expansion and reduced differentiation in IL-7-driven pre-B cell cultures is an intrinsic feature of Btk$^-$ cells, which is independent of the *xid* BM microenvironment in vivo, or coculture of Btk-deficient stromal cells in the BM cultures.

Aberrant phenotype of Btk$^-$ cells in IL-7-driven BM cultures

Next, we investigated whether the inefficient pre-BCR-mediated induction of cell phenotype changes in Btk$^-$ pre-B and immature B cells in vivo were paralleled in the BM cultures in vitro. Large and small IgM$^-$ cells were obtained from total BM cultures in the presence of IL-7 for 7 days. Small IgM$^+$ cells were obtained from total BM cell suspensions that were cultured in the presence of IL-7 for 5 days and subsequently in the absence of IL-7 on S17 stromal cells for 2 days. In agreement with our findings in vivo (Fig. 1), we found by flow cytometric analysis that in Btk$^-$ cultures down-regulation of CD43 and up-regulation of CD2 and CD25 at the large to small pre-B cell transition was impaired (Fig. 5). The expression of BP-1 was elevated in all three stages analyzed. Most importantly, we noticed that in the absence of Btk, SLC synthesis is not efficiently turned off, resulting in significantly enhanced cytoplasmic and surface SLC expression in Btk$^-$ large pre-B cells (96 $\pm$ 1% and 29 $\pm$ 2%, $n = 3$, respectively), compared with 67 $\pm$ 5% and 2.9 $\pm$ 0.9% ($n = 3$) in Btk$^+$ cells. Also in small pre-B cells, cytoplasmic SLC expression was enhanced in Btk$^-$ cultures (62 $\pm$ 5%), compared with Btk$^+$ cultures (17 $\pm$ 3%;

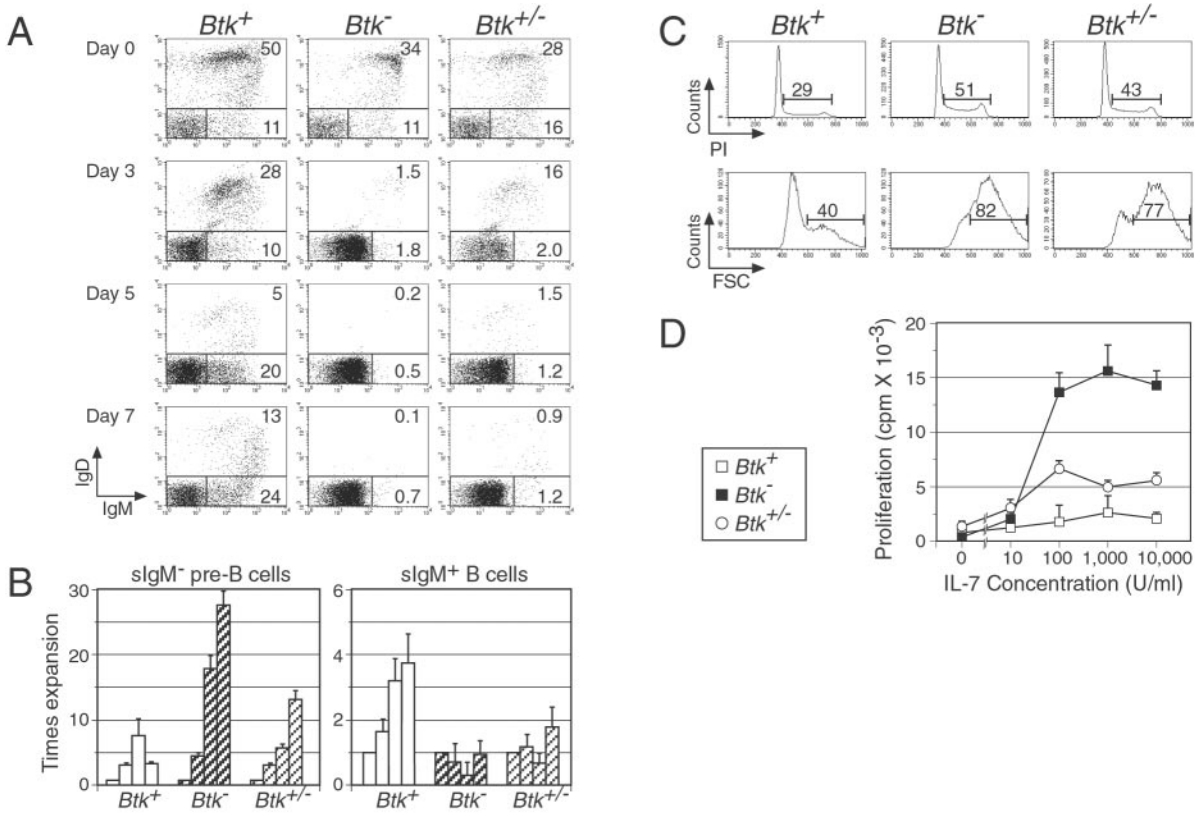

FIGURE 4. Analysis of IL-7-driven BM cultures from Btk$^+$, Btk$^-$, and Btk$^{+/-}$ heterozygous female mice. *A*, IgM/IgD expression profile of B220$^+$ cells in uncultured BM samples, as well as after the indicated days of culture in the presence of IL-7. The gates for the pre-B cells (sIgM$^{-/low}$), immature B cells (IgM$^+$IgD$^-$), and mature B cells (IgD$^+$) are indicated, as well as the percentages of cells within the B cell gates. Note that the pre-B vs immature B gate settings for Btk$^+$ and Btk$^-$ mice at days 3, 5, and 7 are almost one log different, as they are based on differential expression of κ, λ, and SLC in parallel stainings (see *Results*). Dot plots are representative of 8–11 mice per group. *B*, Expansion of IgM$^-$ pre-B cells and IgM$^+$ B cells during culture with IL-7. The four bars per group indicate the expansion at days 3, 5, and 7, as compared with the IgM$^-$ and IgM$^+$ (pre-)B cell numbers at the start of the culture, which were set to one. The plot is representative of six experiments using 2–4 mice per group. Error bars are the SD from three mice within every group. *C*, Cell cycle status and cell size after 5 days of culture with IL-7 from Btk$^+$, Btk$^-$, and Btk$^{+/-}$ mice. DNA content of total cultured cells was examined by propidium iodide staining. The percentages of cycling cells (S, M, or G_2 phase) are indicated. Cell size was examined in B220$^+$IgM$^-$IgD$^-$ pre-B cells. Numbers indicate the percentage of cells with a FSC value >600. Histograms are representative of five to six mice per group. *D*, Proliferative responses to the indicated concentrations of IL-7, as determined by [^{3}H]thymidine incorporation after 5 days of culture. Error bars are the SD from three mice within every group. The plot is representative of three experiments.

Fig. 5). A small but reproducible increase in the expression level of cμ was present in Btk-deficient large and small pre-B cells. In addition, significant fractions of cytoplasmic κ L chain-positive pre-B cells were only present in Btk$^+$ cultures (~20% of small sIgM$^-$ pre-B cells, compared with <2% in Btk-deficient pre-B cells).

Taken together, these findings in IL-7-driven BM cultures confirm the involvement of Btk signaling in the pre-BCR-mediated down-regulation of CD43 and SLC and the induction of CD2 and CD25 expression, and the initiation of Ig L chain rearrangements.

Btk$^-$ pro-B cells show significantly increased IL-7-driven proliferation in vitro

To investigate whether Btk-dependent modulation of IL-7 responsiveness in the mouse is specific for the pre-B cell stage, or alternatively, also occurs in earlier stages of B cell development, we crossed the Btk-deficient mice onto a RAG-1$^{-/-}$ background. In these mice, B cell development is arrested at the CD43$^+$ pro-B cell stage, in which the BCR-linked CD79α/CD79β heterodimers are expressed on the cell surface without associated μ H chains (39). Btk$^+$ and Btk$^-$RAG-1$^{-/-}$ mice contained similar numbers of CD43$^+$B220$^+$ pro-B cells in vivo (data not shown), but the expression of IL-7R was slightly different (~62% in Btk$^+$ and ~79% in Btk$^-$RAG-1$^{-/-}$ mice). In [^{3}H]thymidine incorporation experiments, IL-7-induced proliferation was significantly enhanced in Btk$^-$RAG-1$^{-/-}$ pro-B cells, when compared with control Btk$^+$RAG-1$^{-/-}$ pro-B cells (Fig. 6*A*). Whereas we did not detect significant differences between the two groups of mice in the in vivo expression of the BP-1, the expression was elevated in the Btk$^-$RAG-1$^{-/-}$BM cultures (Fig. 6*B*). Collectively, these results indicate that Btk-signaling can modulate the responsiveness to IL-7 of early B cell precursors, even before pre-BCR expression.

Discussion

Btk and pre-BCR signaling

Signaling through the pre-BCR complex mediates the checkpoint function of μ H chain by inducing cell cycle entry and rapid down-regulation of the rearrangement machinery, thereby ensuring allelic exclusion (14, 29). Subsequently, the proliferating pre-B cells exit the cell cycle, change the expression profile of various cell surface markers, and perform Ig L chain rearrangements with sequential activation of the κ and the λ loci (14). In humans, mutations in the *Btk* gene result in XLA, which reflects an almost complete block in B cell development at the pre-B cell stage (12, 13, 40). Similar to findings in mice deficient for the

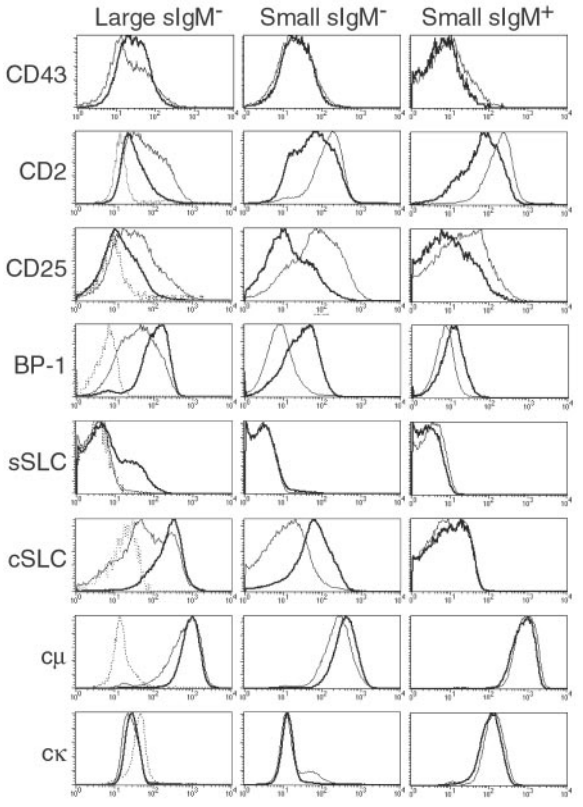

FIGURE 5. Phenotypic characterization of IL-7-driven BM cultures from Btk$^+$ and Btk$^-$ mice. The expression profiles of the indicated surface and intracellular markers are displayed as histogram overlays of Btk$^+$ (*thin lines*) and Btk$^-$ mice (*bold lines*). The histogram plots of the first column (the large sIgM$^-$ cells) include the background stainings of B220$^-$ (S17 stromal) cells, as negative controls (*dotted lines*). This is not shown for CD43, as the B220$^-$ fraction in the cultures expressed CD43. Large and small IgM$^-$ cells were gated on the basis of FSC characteristics. Results shown are representative of 2–6 mice per group.

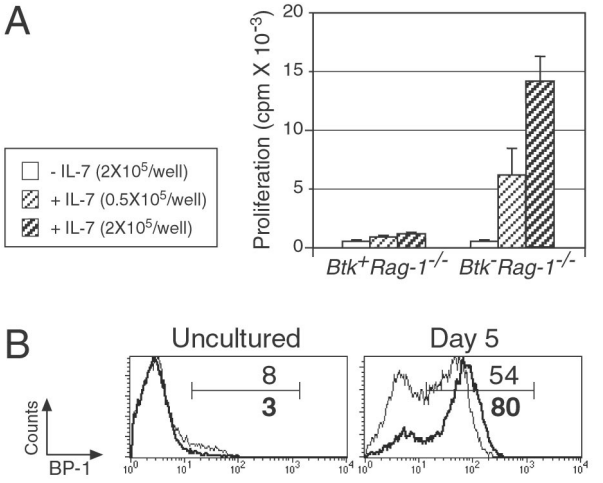

FIGURE 6. Btk affects IL-7 responsiveness in pro-B cells. *A*, Proliferative responses of Btk$^+$ and Btk$^-$ RAG-1$^{-/-}$ B cell precursors to IL-7, as determined by [^{3}H]thymidine incorporation after 7 days of culture. *B*, BP-1 expression profiles are displayed as histograms for B220$^+$ cells, either uncultured or after 5 days of culture in the presence of IL-7, from Btk$^+$ (*thin lines*) and Btk$^-$ (*bold lines*) RAG-1$^{-/-}$ BM. Data are representative of four experiments using two mice per group.

SLC component λ5 or the membrane form of the Ig μ H chain (41, 42), most XLA patients lack the compartment of large cycling pre-B cells (13). Therefore, the XLA disease phenotype implicates Btk in the pre-BCR signaling-mediated expansion of those pre-B cells with a productive μ H chain rearrangement in humans.

In contrast, Btk is not essential for the selective expansion of cμ$^+$ pre-B cells in the mouse, as in Btk-deficient mice the pre-B cell population is normal in size and contains normal proportions of cycling cells (16–19). In this report, we demonstrate that the mouse Btk is involved in later phases of pre-B cell differentiation. We compared the pre-B cell compartment in Btk$^+$ mice and Btk$^-$ mice both in vivo and in vitro in IL-7-driven BM cultures. The absence of Btk resulted in 1) defective down-regulation of CD43 and SLC expression, 2) impaired up-regulation of CD2 and CD25, 3) aberrant expression of BP-1, 4) a 3-h delay in B cell development in vivo at the small pre-B cell stage and reduced progression to surface Ig$^+$ B cells in vitro, and 5) a significant increase in IL-7-driven proliferative expansion of pro-B and pre-B cells in vitro. Because Btk has been reported to be in the pre-BCR signaling pathway in the mouse (5, 22), our findings implicate pre-BCR signaling in the control of CD2, CD25, CD43, BP-1, and SLC expression, IL-7 responsiveness, and the transit time through the small pre-B cell stage.

As pre-B cell production in Btk$^-$ mice is normal, we conclude that the inefficient down-regulation of BP-1, CD43, and SLC expression and up-regulation of CD2 and CD25 do not affect the proliferative capacities of pre-B cells in vivo. Nevertheless, Btk$^-$ large pre-B cells manifested increased proliferative expansion in vitro, when compared with Btk$^+$ cells. The finding of significantly increased surface SLC expression on these Btk$^-$ large pre-B cells (when compared with Btk$^+$ cells) would support the hypothesis by Melchers et al. (14) that large cμ$^+$ pre-B cells stop cycling when they run out of a sufficient number of assembled pre-BCR molecules per cell. This is not necessarily in conflict with our finding that the increased cytoplasmic SLC expression in Btk$^-$ pre-B cells did not result in increased proliferation in vivo. It is very possible that only the level of membrane SLC expression may correlate with the proliferative capacity of pre-B cells, and we were unable to detect differences between Btk$^+$ and Btk$^-$ large pre-B cells in vivo in membrane SLC expression levels.

Btk and L chain rearrangement

We have previously found that Btk$^-$ mice show reduced λ L chain usage, which could either result from a defect in receptor editing or alternatively from a decreased rate or efficiency of λ L chain rearrangement (20). Although by crossing 3-83 μδ autoantibody transgenic mice into Btk$^-$ mice we have shown that Btk is not essential for receptor editing (20), a possibility remained that the extent of L chain replacement events is reduced in Btk$^-$ mice. However, our BrdU-labeling experiments, in which we identified a 3-h delay at the small pre-B cell stage in Btk$^-$ mice, clearly argue against this possibility. Reduction of the level of receptor editing in Btk$^-$ mice would have the opposite effect of a more rapid transit through the small pre-B cell compartment, as B cells targeted for receptor editing were recently shown to be specifically delayed in this compartment for at least 2 h (33). Therefore, the finding of a 3-h delay supports a role for Btk in the regulation of the initiation of L chain rearrangement events. As both κ$^+$ and λ$^+$ Btk$^-$ cells are equally delayed, this cannot explain the observed reduced λ usage in Btk$^-$ mice (20). However, we conclude that the absence of Btk specifically affects the production rate of λ$^+$ immature B cells, as our linear regression analysis (Fig. 2*C*) revealed a lower slope value for λ$^+$ cells in Btk$^-$ mice (1.3) than in Btk$^+$ mice (1.7), while the values for κ$^+$ cells were similar (1.2 and 1.3).

In this context, it is unknown whether Btk signaling directly or indirectly regulates the activation of the L chain loci, or alternatively, whether Btk acts by mediating developmental progression to a pre-B cell stage in which L chain rearrangements are initiated. The latter possibility would be consistent with the delayed modulation of the expression of SLC, BP-1, CD43, CD2, and CD25. The finding that the expression of germline κ transcripts is first detected in vivo in large cycling $CD25^+$ pre-B cells would be consistent with a role for pre-BCR signaling the opening of the L chain loci for rearrangement (29, 43). However, additional experiments would be needed to address this issue, as it has been shown that productive L chain rearrangement can also be induced by IL-7 withdrawal of pre-BCR-deficient $\lambda 5^{-/-}$ or $J_H{}^{-/-}$ pre-B cells in vitro (14, 44).

The size of the immature B cell pool and the turnover of immature B cells were reported to be essentially normal in *xid* or Btk^- mice (16–18, 45). Nevertheless, we previously observed a selective disadvantage of Btk^- cells at the transition from pre-B cells to immature B cells in the BM, which we determined in an in vivo competition analysis in $Btk^{+/-}$ heterozygous female mice (17). In the immature B cell subset, we found *lacZ* expression values of ~30%, whereas in the absence of a competitive disadvantage, $Btk^-/lacZ^+$ cells would be expected to represent ~50% of any B cell subpopulation. The finding of a 3-h delay in production of immature B cells in Btk^- mice would explain the observed selective disadvantage of Btk-deficient cells in $Btk^{+/-}$ female mice.

Btk and IL-7 responsiveness

An inhibitory effect of Btk on proliferation of B-lineage cells in long-term Whitlock-Witte BM cultures has previously been reported (46). However, analysis of growth kinetics of cultures established from mixtures of WT and *xid* cells indicated that the observed differences resulted from changes in the BM microenvironment associated with the *xid* mutation. In contrast, our separate analyses of $lacZ^+$ and $lacZ^-$ cells in the BM cultures from $Btk^{+/-}$ mice showed that increased IL-7 responsiveness is an intrinsic feature of Btk^- pre-B cells. This would be consistent with the finding of an increased frequency of CFU responsive to IL-7 by limiting dilution analysis of day 15 fetal liver cells from CBA/N *xid* mice (47).

Finally, crosses of Btk-deficient and RAG-1-deficient mice showed that IL-7-dependent expansion in vitro is constrained by an inhibitory signal mediated by Btk, even before the expression of μ H chain. Cross-linking of the Igβ signaling component of the (pre-)BCR, which is expressed on the cell surface in $c\mu^-$ pro-B cells in association with calnexin induced a rapid phosphorylation of several intracellular signaling molecules, including Btk (22, 48). Therefore, it is possible that Btk-mediated Igβ/calnexin signaling would serve to stop pro-B cell proliferation to facilitate V(D)J recombination, which is exclusively initiated in the G_0/G_1 cell cycle phase (49). This hypothesis may be supported by a significant reduction of the level of V_H to DJ_H rearrangements observed in Igβ-deficient mice (50), which would then be explained by a reduced signal to make pro-B cells competent to undergo V_H-DJ_H rearrangement (48). In this context, it has been shown previously that in later stages of B cell development, modulation of the IL-7 responsiveness is dependent on pre-BCR and BCR signaling (51, 52). However, it is very possible that in pro-B cells, Btk may function in a pathway that is unrelated to the Igβ/calnexin complex, as Btk has been implicated in signaling of various other receptors, including IL-5R, CD38, IL-10R, and FcϵRI (1, 2).

Taken together, our findings implicate Btk in two different pathways in early B cell development. First, Btk is required for the efficient developmental progression of cytoplasmic μ^+ pre-B cells, as Btk was shown to be involved in BP-1, CD43, and SLC down-regulation, CD2 and CD25 up-regulation, and the rate of transit through the small pre-B cell compartment. Additional experiments are required to show whether Btk-mediated pre-BCR signaling directly or indirectly influences the expression of SLC, germline κ, or λ transcripts or the RAG genes. Second, the IL-7-driven BM culture experiments indicate a role for Btk as a pro- and pre-BCR associated negative regulator of IL-7 expansion. Although XLA patients may occasionally have a significantly increased pro-B cell compartment (12, 53), in general there is no absolute pro-B expansion in XLA. Obviously, both in the mouse and in humans, other signaling molecules or pathways must be able to compensate for the loss of Btk in pro-B cells in vivo.

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