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Original Article

Bruton's tyrosine kinase mediated signaling enhances leukemogenesis in a mouse model for chronic lymphocytic leukemia

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Abstract: In chronic lymphocytic leukemia (CLL) signals from the B cell receptor (BCR) play a major role in disease development and progression. In this light, new therapies that specifically target signaling molecules downstream of the BCR continue to be developed. While first studies on the selective small molecule inhibitor of Bruton's tyrosine kinase (Btk), Ibrutinib (PCI-32765), demonstrated that Btk inhibition sensitizes CLL cells to apoptosis and alters their migratory behavior, these studies however did not address whether Btk-mediated signaling is involved in the process of CLL leukemogenesis. To investigate the requirement of Btk signaling for CLL development, we modulated Btk expression in the IgH.ETμ CLL mouse model, which is based on sporadic expression of the simian oncovirus SV40 T-antigen in mature B cells. To this end, we crossed IgH.ETμ mice on a Btk-deficient background or introduced a human Btk transgene (CD19-hBtk). Here we show that Btk deficiency fully abrogates CLL formation in IgH.ETμ mice, and that leukemias formed in Btk haplo-insufficient mice selectively expressed the wild-type Btk allele on their active X chromosome. Conversely, Btk overexpression accelerated CLL onset, increased mortality, and was associated with selection of non-stereotypical BCRs into CLL clones. Taken together, these data show that Btk expression represents an absolute prerequisite for CLL development and that Btk mediated signaling enhances leukemogenesis in mice. We therefore conclude that in CLL Btk expression levels set the threshold for malignant transformation.

Keywords: Chronic lymphocytic leukemia (CLL), bruton's tyrosine kinase (Btk), B cell receptor signaling

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world with incidence rates as high as ~4 per 100.000 individuals in the USA [1]. Approximately half of the CLL cases carry unmutated immunoglobulin heavy (IgH) chain variable (V) regions, which is associated with an unfavorable prognosis (see for review: Kil *et al*. [2]). Recently reported transcriptome analyses of CLL and normal B cell subsets revealed that unmutated CLL derives from unmutated mature $CD5^+CD27^-$ B cells and mutated CLL derives from a distinct, previously unrecognized, $CD5^+CD27^+$ post-germinal center B cell subset [3].

In CLL, defects in apoptosis lead to the accumulation of $CD5^+$ mature B lymphocytes that express low levels of surface Ig, suggestive of continuous engagement of the B cell receptor (BCR) by antigens [4, 5]. Such chronic BCR signaling may provide strong anti-apoptotic signals that support both malignant transformation of mature B cells and persistence of these transformed B cells [6]. In support of a central role for chronic BCR signaling in CLL pathogenesis is the high recurrence of stereotypic BCRs among CLL samples, pointing to a probably limited set of allo- or autoantigens that stimulate these stereotypic BCRs [7].

Interestingly, it was very recently reported that most CLL BCRs can recognize an internal BCR epitope that additionally provides an abundant cell-intrinsic source of antigenic stimulation [8]. In line with chronic BCR stimulation, CLL B cells exhibit a distinct BCR signaling profile that

closely resembles signaling of anergic B cells, characterized by incomplete activation of downstream BCR signaling pathways and weaker responses to BCR triggering using soluble antibodies [9, 10]. The importance of continuous BCR signaling for CLL cell survival is further demonstrated by the pronounced pro-apoptotic effects of new drugs that specifically target BCR signaling molecules [2, 11].

A novel CLL therapeutic target downstream of the BCR is Bruton's tyrosine kinase (Btk) [2]. Although widely expressed in hematopoietic cells, except T cells, only B cells greatly rely on Btk for their development, activation and survival, as Btk deficiency in man or mice results in the B cell specific immunodeficiencies X-linked agammaglobulinemia (XLA) or x-linked immune deficiency (*xid*), respectively [12]. The strong reduction of mature B cell numbers in *xid* mainly results from hampered survival of mature B cells, which may largely be explained by the ineffective activation of Btk's primary signaling target NF-κB [13, 14]. Conversely, transgenic B cells overexpressing wild-type human Btk were selectively hyperresponsive to BCR stimulation and showed enhanced NF-κB activation, resistance to Fas-mediated apoptosis and defective elimination of selfreactive B cells *in vivo* [15]. Consistent with the critical role for Btk in B cell survival, CLL cells lose their resistance to apoptosis *in vitro* when treated with the selective Btk-inhibitor PCI-32765 [16, 17]. Furthermore in a murine transfer model the *in vivo* expansion of transplanted CLL cells was markedly reduced by PCI-32765 treatment [17]. These effects of Btk inhibition probably extend beyond dampening of BCR signaling alone, since PCI-32765 treatment also affected CLL cell adhesion, as well as migration directed by CXCR4 and CXCR5 that both employ Btk as downstream signaling molecule [17-20].

Since the levels of Btk represent a rate-limiting step in BCR signaling and thereby B cell activation and survival [15], the recent finding of Btk overexpression in CLL samples [16] prompted us to investigate the influence of Btk expression levels on CLL development. To this end, we used IgH.ETμ mice, a transgenic mouse model that exhibits spontaneous CLL development driven by sporadic expression of the simian oncovirus SV40 T-antigen in mature B cells [21]. In these mice, B-cell development is unperturbed in young mice, but in aging mice $IgD^{low}CD5^{+}$ monoclonal leukemic B cells accumulate with frequent usage of the Ig heavy (IgH) chain V_H11 family. Here, we demonstrate that Btk-deficiency completely abrogated CLL development in IgH.ETμ mice. Conversely, B cell specific overexpression of Btk accelerated CLL development, increased overall CLL incidence, and altered the Ig light (IgL) chain repertoire in CLL.

Materials and methods

Mouse tumor cohorts and genotyping

IgH.TEμ mice [21], Btk-deficient mice [22] and CD19-hBtk transgenic mice [23] were all on the C57BL/6 background for >10 generations and were crossbred to generate IgH.TEμ mice tumor cohorts that were haplo-insufficient or deficient for Btk, or transgenically overexpressing human Btk. The mice were genotyped with a polymerase chain reaction (PCR) using genomic DNA with the following primers (Life Technologies Europe BV) for the IgH.TEμ construct (forward 5'-GGAAAGTCCTTGGGGTCTTC-3', reverse 5'-CACTTGTGTGGGTTGATTGC-3'), *lacZ* (Btk-knockout) alleles (forward 5'-TTCACTGGCCGTCGTTTTACAACGTCGTGA-3', reverse 5'-ATGTGAGCGAGTAACAACCCGTCGGATTCT-3'), wild-type Btk alleles (forward 5'-CACTGAAGCTGAGGACTCCATAG-3', reverse 5'-GAGTCATGTGCTTGGAATACCAC-3'), and the CD19-hBtk transgene (forward 5'-CCTTCCAAGTCCTGGCAT-3', reverse 5'-CACCAGTCTATTTACAGAGA-3'). CLL development in animals in tumor cohorts was monitored every 3-6 weeks by screening peripheral blood for monoclonal B cell expansion using flow cytometry (see below, "General flow cytometry procedures"), and were sacrificed after detection of CLL by severe monoclonal B cell lymphocytosis, or after a maximum period of 60 weeks of disease-free survival. Mice were bred and kept in the Erasmus MC experimental animal facility and the experiments were approved by the Erasmus MC committee of animal experiments.

General flow cytometry procedures

Preparation of single-cell suspensions of lymphoid organs and lysis of red blood cells were performed according to standard procedures [15]. Cells were (in)directly stained in flow cytometry buffer (PBS, supplemented with 0.25% BSA, 0.5 mM EDTA and 0.05% sodium

azide) using the following fluorochrome or biotin-conjugated monoclonal antibodies or reagents: anti-B220 (RA3-6B2, eBioscience), anti-CD19 (ID3, eBioscience), anti-CD5 (53-7.3, eBioscience), anti-CD43 (R2/60, eBioscience), anti-CD138 (281-2, BD biosciences), anti-CD95 (Jo2, BD biosciences), anti-IgD (11-26, BD biosciences), anti-IgMb (AF6-78, BD biosciences), anti-IgMa (DS-1, BD Biosciences), anti-Igλ (R26-46, BD biosciences), anti-Igκ (187.1, BD biosciences), anti-CD21 (7G6, BD Biosciences), anti-CD23 (B3B4, eBiosciences), PNA (Sigma-Aldrich), using conjugated streptavidin (eBioscience) as a second step for biotin-conjugated antibodies. For measurement of intracellular Btk levels, cells were fixed in PBS/2% paraformaldehyde (PFA) and permeabilized with permeabilization buffer (0.5% saponin (Sigma-Aldrich) in flow cytometry buffer). For staining of Btk, cells were incubated for 1 hour at room temperature with PE-conjugated anti-Btk (53/BTK, BD Biosciences) in permeabilization buffer. All flow cytometric measurements were performed on an LSRII flow cytometer (BD Biosciences), and prior to measurement cells were washed and resuspended in flow cytometry buffer.

Flow cytometric detection of Erk and Akt phosphorylation

CLL cells and wild-type splenic B cells were purified by magnetic-activated cell sorting (MACS) with anti-CD19 coated magnetic beads (Miltenyi Biotec). Sorted cells were starved for 30 minutes at 37°C in FCS-free "RPMI-plus" medium (RPMI media 1640, supplemented with penicillin-streptomycin, 1.2 mM L-glutamine, non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol; all components from Life Technologies™) and subsequently stimulated with 20 μg/mL goat anti-mouse Igκ (SouthernBiotech) for 5 min. After stimulation, cells were fixed for 10 minutes in 2% PFA, washed with PBS, permeabilized with ice cold 70% methanol in PBS for 30 minutes, and stained with fluorochrome-conjugated anti-Akt(pS473) and anti-Erk1/2(pT202/pY204) antibodies (BD Biosciences).

Evaluation of lacZ gene (β-galactosidase) expression

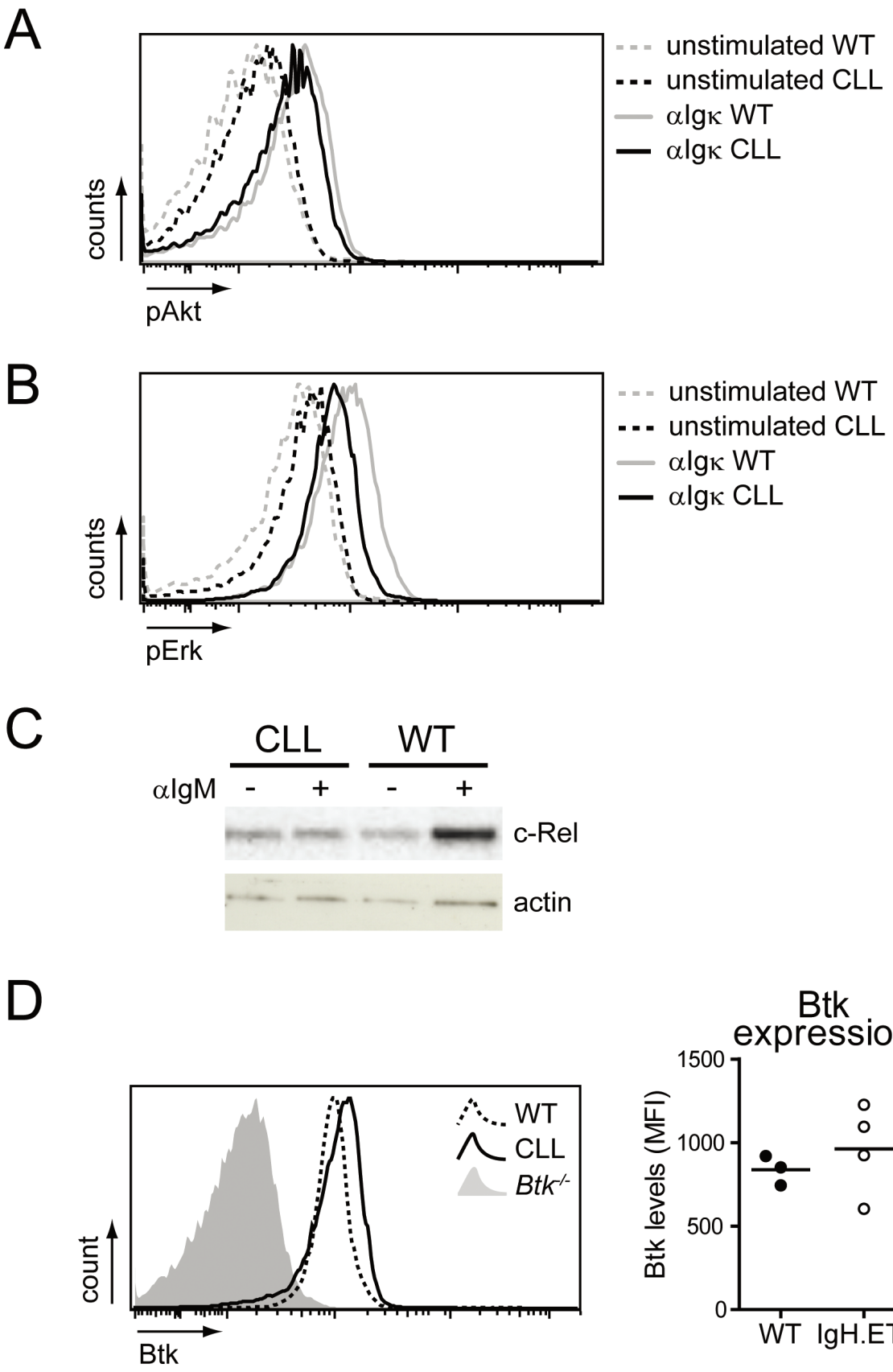
Expression of the *lacZ* cassette (encoding β-galactosidase) incorporated in Btk knockout alleles was evaluated essentially as published in Hendriks *et al*. [22]. Briefly, CLL cells or splenocytes were loaded with fluorescein-di-β-D-galactopyranoside (FDG; Molecular Probes) using a hypotonic shock at 37°C. FDG loading was stopped after 1 minute by washing the cells with ice-cold culture medium, and leaving them for >2 hours on ice to facilitate FDG degradation while minimizing fluorescein leakage from the cells. Further staining for membrane markers was performed on ice during this incubation step, as described above in "General flow cytometric procedures", and fluorescein levels in conjunction with membrane marker expression were evaluated on an LSRII flow cytometer (BD Biosciences).

Measurement of c-Rel nuclear translocation

CLL cells and wild-type splenic B cells were purified as described (see "Flow cytometric detection of Erk and Akt phosphorylation") and were stimulated with goat anti-mouse [F(ab')$_2$] α-IgM fragments (JacksonImmunoResearch) at 37°C in culture medium for 4 hours. Total nuclear protein extracts were prepared and processed as described previously [24]. After washing in PBS, cell nuclei were obtained by incubating cells for 10 minutes on ice in hypotonic Buffer A (10 mM HEPES-KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5.mM DTT, 0.2 mM protease inhibitor PMSF) followed by vortexing. After centrifugation of nuclei, nuclear lysates were prepared by 5 minutes incubation on ice with lysing Buffer C (10 mM HEPES-KOH, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) followed by centrifugation, after which the supernatant was stored at -80°C. Samples were resolved using SDS-PAGE and transferred to nitrocellulose membranes according to standard procedures. Membranes were stained with rabbit anti-mouse c-Rel (Santa Cruz Biotechnology, Inc.) or mouse anti-actin (Chemicon International) primary antibodies and peroxidase-coupled swine anti-rabbit Ig or rabbit anti-mouse Ig secondary antibodies (Dako).

Immunoglobulin (Ig) heavy and light chain sequence analysis

From CLL tumor material total RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's protocol, and cDNA was made using the RevertAid H minus First Strand


A
unstimulated WT
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B
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pErk
C
CLL
WT
αIgM - + - +
c-Rel
actin
D
WT
CLL
Btk-/-
count
Btk
Btk expression
1500
1000
500
0
Btk levels (MFI)
WT
IgH.ETμ

Figure 1. Altered activation of downstream BCR signaling pathways in IgH.ETμ tumors. Flow cytometric measurement of phosphorylation of Akt (A) and Erk (B) upon BCR stimulation in IgH.ETμ CLL cells versus wild-type (WT) B cells. MACS-purified $CD19^+$ cells were stimulated for 5 minutes with 20 μg/mL goat anti-mouse Igκ and subsequently stained for phosphorylated Akt or Erk. C: Protein levels of c-Rel determined by western blotting in nuclear lysates of unstimulated (-) and αIgM stimulated (+) $CD19^+$ MACS-sorted CLL cells and wild-type (WT) splenic B cells. D: Left: Btk expression levels in gated $CD19^+$ wild-type (WT) splenic cells versus IgH.ETμ CLL cells were evaluated using flow cytometry. Background staining levels were determined in *Btk*$^{-/-}$ cells. Right: flow cytometric determination of median Btk expression levels in wild-type (filled circles) and IgH.ETμ CLL cells (open circles). MFI: median fluorescence intensity. For all analyses at least 4 CLL samples and 3 wild-type splenic B cell fractions were included; representative results are shown.

cDNA sythesis kit (Thermo Scientific). For PCR amplification of the Ig heavy chain, two FR1 region high-degeneracy primers MH1 and MH2 [25] or one high-degeneracy primer IPP000009 (IMGT®, http://www.imgt.org) were combined with a primer targeting the 5' cμ region [26, 27]. Primers used for PCR amplification of the Ig kappa [25] and Ig lambda light chains [28] have previously been described. PCR products were directly sequenced with these primers using the BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase on an ABI 3130xl automated sequencer (Applied Biosystems). Sequences were analyzed using CLC DNA workbench (CLCbio) and blasted in IMGT/V-Quest (http://www.imgt.org, using Ig gene nomenclature as provided by IMGT on Nov 1st 2012), and all sequences were confirmed in at least one duplicate analysis.

Statistical analysis

For calculating levels of significance, the student's t test was used for differences between groups of continuous data, whereas Fisher's exact test was used for differences between group proportions. The log rank test was used for calculating the level of significance for incidence and survival differences between tumor cohorts.

Results

Incomplete activation of BCR signaling pathways in IgH.ETμ CLL cells

There is substantial evidence for a significant contribution of Btk signaling to CLL formation or progression [16, 17, 20], but it remains unclear whether Btk signaling mainly exerts its pathogenic role downstream of the BCR, or downstream of other receptors, including chemokine receptors. We therefore aimed to characterize the engagement of different signaling pathways downstream of the BCR that are known to be activated rather independent of Btk (Akt), partially dependent on Btk (Erk), or greatly dependent on Btk signals (NF-κB) [2]. To this end, we compared activation of these downstream signaling molecules in purified wild-type splenic B cells and primary mouse CLL cells isolated from diseased IgH.ETμ mice. In unstimulated IgH.ETμ CLL cells we observed a very low basal phosphorylation level of both Akt and Erk, which was slightly higher than in normal splenic B cells (**Figure 1A** and **1B**). Upon brief BCR stimulation *in vitro*, Akt phosphorylation in IgH.ETμ CLL cells was almost indistinguishable from wild-type splenic B cells (**Figure 1A**), whereas phosphorylation of Erk was weaker in stimulated IgH.ETμ CLL cells (**Figure 1B**). In contrast to the normal or suboptimal activation of Akt and Erk respectively, we could not detect induction of phosphorylated Btk or any nuclear translocation of NF-κB (c-Rel) upon prolonged BCR stimulation of IgH.ETμ CLL cells *in vitro* using western blotting (**Figure 1C** and data not shown).

The weak activation of downstream BCR signaling targets of Btk prompted us to investigate whether Btk expression levels were subphysiological in IgH.ETμ CLL cells, since Btk expression levels are tightly regulated upon stimulation by the BCR or other activating receptors, through NF-κB and miR185-mediated pathways [15, 29-31]. Using intracellular flow cytometry, we measured Btk expression levels in primary IgH.ETμ CLL cells in comparison to wild-type splenic B cells. We found that Btk expression levels in IgH.ETμ CLL cells were more variable than Btk levels in non-malignant B cells. We did not find evidence for a decrease in Btk expression, since two out of four CLL samples analyzed displayed Btk overexpression (**Figure 1D**).

These results demonstrate aberrant BCR signaling in primary CLL cells compared with wild-type B cells and furthermore indicate that Btk

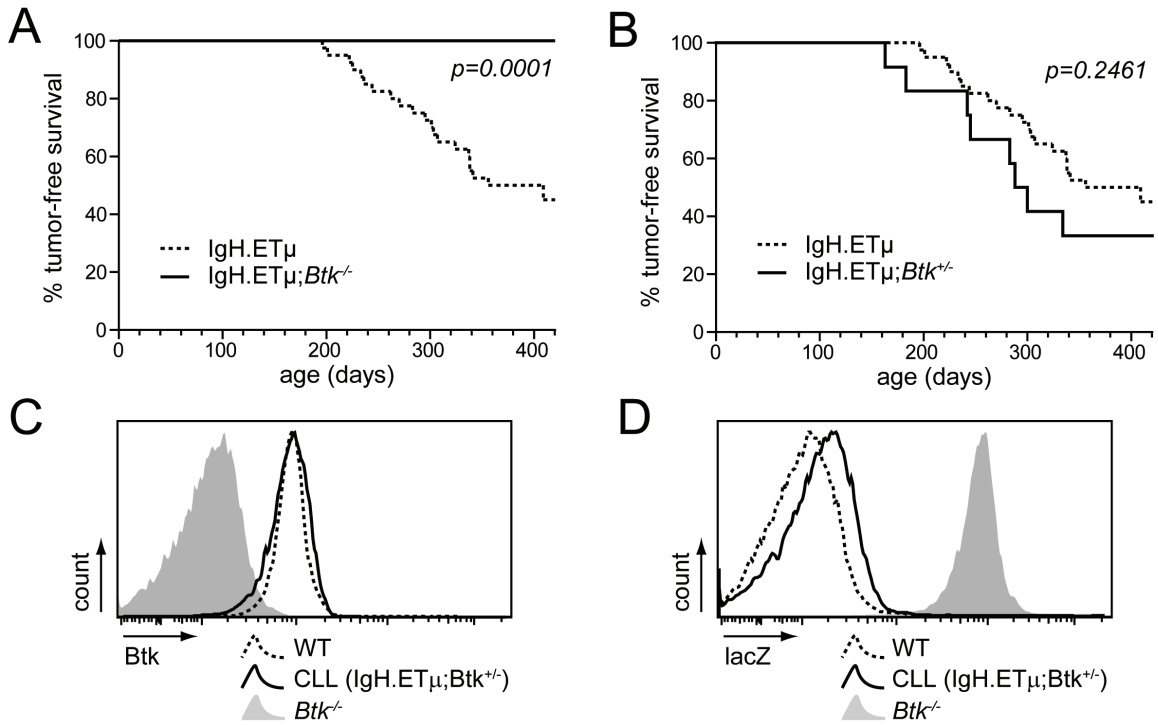

Figure 2. Btk is essential for CLL development. A: Kaplan-Meier survival curves of IgH.ETμ mice (dotted line; n=40) and Btk-deficient IgH.ETμ littermates (continuous line; n=19). B: Kaplan-Meier survival curves of IgH.ETμ mice (dotted line; n=40) and Btk-haplo-insufficient IgH.ETμ littermates (continuous line; n=12). C: Comparison of Btk expression levels in IgH.ETμ;*Btk*$^{+/-}$ CLL cells as in Figure 1D. D: Flow cytometric measurement of β-galactosidase expression from *lacZ*-inserted Btk-knockout alleles in IgH.ETμ;*Btk*$^{+/-}$ CLL cells versus *Btk*$^{-/-}$ B cells, as determined by release of fluorescein upon FDG substrate degradation. Wild-type (WT) B cells were used as negative control. In (C) and (D) representative results are shown of 4 CLL samples analyzed.

activation downstream of the BCR is weak and associated with incomplete activation of known Btk signaling targets, particularly NF-κB.

CLL development is fully dependent on Btk

To test whether Btk signaling is critical for the formation of CLL, we monitored CLL development in cohorts of Btk-deficient IgH.ETμ mice versus Btk-sufficient IgH.ETμ mice, which were backcrossed onto the C57bl/6 genetic background for >10 generations. In contrast to the 100% CLL incidence and median survival of 161 days reported in IgH.ETμ mice on a mixed C57bl/6 x 129/Sv genetic background [21], we now observed a 55% CLL incidence with a median survival of 356 days in this cohort (**Figure 2A**). Strikingly, in none of the 19 IgH.ETμ mice on a Btk-deficient background we observed CLL formation (**Figure 2B**), nor did we detect any other expansion of monoclonal B cells with or without CLL-like features in peripheral blood samples that were obtained from these mice every 3-6 weeks. We also did not detect increased cellularity in lymphoid organs, including lymph nodes, spleen and bone marrow, after sacrificing mice at 60 weeks of age.

To further confirm the absolute requirement for Btk expression in CLL development, we followed CLL development in IgH.ETμ females that were haplo-insufficient for Btk. The *Btk* gene is located on the X-chromosome, and random X-inactivation occurring in all female somatic cells will therefore stochastically restrict Btk expression to one allele. In *Btk*$^{+/-}$ females this results in the generation of B cell precursors in the bone marrow that either express the normal Btk gene from the wild-type X chromosome, or express the *lacZ* reporter inserted in the targeted Btk allele from the mutated X chromosome [22]. In peripheral B cells in *Btk*$^{+/-}$ female mice the distribution of Btk versus *lacZ*-expressing mature B cells is however largely skewed towards Btk-expressing cells, since lack of Btk expression poses a selective disad-

vantage for circulating immature B cells. Nevertheless, ~30% of $IgM^{high}IgD^{low}$ immature B cells in *Btk*$^{+/-}$ females do not carry the wild-type *Btk* locus on their active X chromosome and thus are Btk-deficient [22].

In IgH.ETμ;*Btk*$^{+/-}$ mice (n=12) we observed a CLL incidence comparable to IgH.ETμ mice (67% versus 55%, p=0.2461; see **Figure 2B**). In agreement with this finding, flow cytometric analysis for intracellular Btk in all IgH.ETμ;*Btk*$^{+/-}$ CLL samples revealed normal Btk expression in these tumors (**Figure 2C**) and accordingly no *lacZ* expression from the Btk mutant allele was detected (**Figure 2D**). Thus, in IgH.ETμ;*Btk*$^{+/-}$ mice only Btk-expressing cells were susceptible to CLL development by SV40 large T antigen mediated oncogenic transformation.

Taken together, these experiments show that Btk expression is an absolute prerequisite for CLL development in mice.

High-level Btk expression enhances CLL formation

Although variable overexpression of Btk is observed both in human CLL [16] and in our IgH.ETμ CLL samples (**Figure 1D**), it is unclear whether increased expression levels of Btk may increase the risk of CLL development or promote disease progression in CLL. To investigate this, we generated IgH.ETμ mice that overexpress a human Btk transgene selectively in the B-cell lineage under the control of the CD19 promoter region (CD19-hBtk) [23]. We recently found that enhanced BCR signaling through Btk increases B cell activation and promotes B cell survival [15]. When CD19-hBtk transgenic mice were followed over time up to 1 year of age, we did not find evidence for spontaneous B cell malignancies, but instead observed selective persistence of self-reactive B cells [15]. In aging mice, we detected spontaneous differentiation into germinal center B cells and plasma cells *in vivo*, associated with auto-antibody formation and an SLE-like auto-immune disease. CD19-hBtk transgenic B cells were selectively hyperresponsive to BCR stimulation and showed enhanced αIgM-induced Ca^{++} influx and NF-κB activation [15]. Btk overexpression did not affect *in vitro* vascular cell adhesion molecule-1 (VCAM1) mediated migration of B cells in response to CXCL12 or CXCL13 (data not shown), all of which is impaired in the absence of Btk [18-20].

Follow-up of a cohort of IgH.ETμ;CD19-hBtk mice demonstrated an earlier onset of CLL formation (median onset at 152 days, compared with 243 days in IgH.ETμ control mice) as well as an increase in CLL incidence (85% versus 55%, p<0.0001; **Figure 3A**). Moreover, the mortality was higher in IgH.ETμ;CD19-hBtk mice and the median survival was decreased compared to IgH.ETμ mice (315 versus 356 days). We noticed, however, a larger average interval between CLL diagnosis and CLL-associated death in IgH.ETμ;CD19-hBtk mice (151 versus 67 days; **Figure 3**).

Taken together, these data show that Btk-mediated signaling enhances CLL lymphomagenesis in mice.

High-level Btk expression is associated with CLL with non-stereotypic BCRs

To determine whether enhanced Btk-mediated signaling may lower the threshold for BCR-based selection of certain B cells into CLL clones, we characterized the BCRs expressed by CLLs from IgH.ETμ, IgH.ETμ;*Btk*$^{+/-}$ and Btk-overexpressing IgH.ETμ;CD19-hBtk mice. Flow cytometric and BCR sequencing analyses showed that 35% of IgH.ETμ;CD19-hBtk tumors expressed an Ig lambda (Igλ) light chain, compared with only 4% of tumors from IgH.ETμ and IgH.ETμ;*Btk*$^{+/-}$ mice (p=0.01; **Figure 4A**). This cannot be explained by an effect of Btk overexpression on Igλ usage in normal B cells, because in young non-diseased CD19-hBtk mice the proportion of Igλ expressing mature B cells was not increased: ~9.2% in CD19-hBtk mice versus ~8.5% in wild-type mice.

DNA sequence analysis of IgH, Igκ and Igλ chains revealed that 36% of tumors (9 out of 25) from IgH.ETμ and IgH.ETμ;*Btk*$^{+/-}$ mice were highly stereotypical, using only one combination of V_H11-2 and $V_κ$14-126 genes and expressing a largely similar IgH complementarity-determining region 3 (CDR3) motif (**Table 1**, **Figure 4B**). In IgH.ETμ;CD19-hBtk mice we identified V_H11-2/ $V_κ$14-126 usage in 19% (3 out of 16) of CLL samples, indicating an increase in non-stereotypical BCR usage (**Table 1**, **Figure 4B**). The cohorts of IgH.ETμ mice on the C57bl/6 background showed infrequent development of

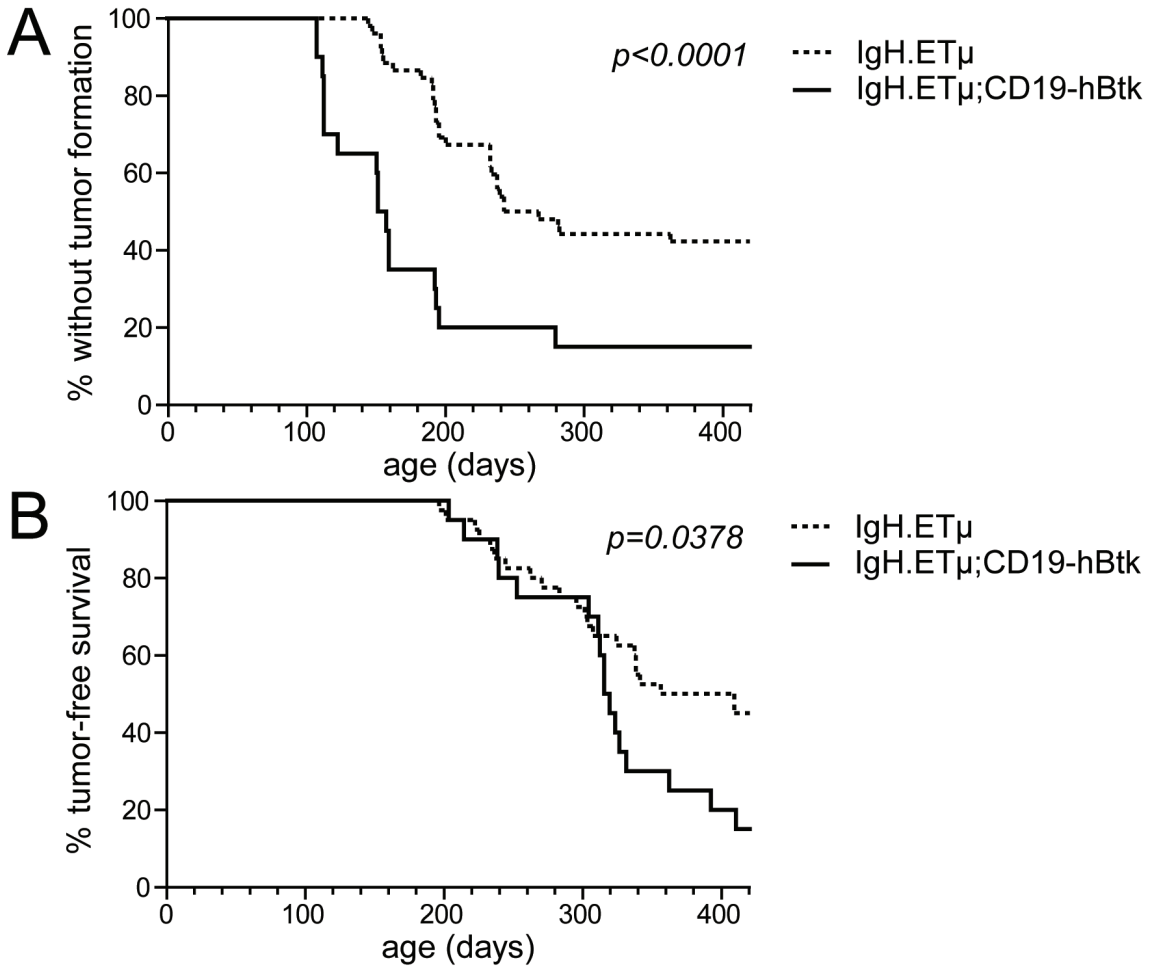

Figure 3. Btk overexpression enhances CLL formation. A: Incidence curve of CLL formation in IgH.ETμ mice (dotted line; n=40) versus CD19-hBtk transgenic IgH.ETμ mice (continuous line; n=20). CLL formation was defined by the first appearance of monoclonal $IgMb^+$ B cells in peripheral blood of these mice ($IgMb^+$:$IgMa^+$ ratio > 80:20 [21]). B: Kaplan-Meier survival curve of IgH.ETμ;CD19-hBtk mice (continuous line; n=20) versus IgH.ETμ non-CD19-hBtk transgenic mice (dotted line; n=40).

mutated CLLs (M-CLLs) that express BCRs harboring >2% mutated nucleotides (2 out of 25 tumors) and no significant difference in M-CLL occurrence was noted in IgH.ETμ;CD19-hBtk mice (none out of 17 tumors). Further CDR3 characterization of IgH from tumors in IgH.ETμ;CD19-hBtk mice revealed a significant increase in CDR3 length, compared with IgH.ETμ and IgH.ETμ;*Btk*$^{+/-}$ mice (p=0.0394, **Figure 4C**). In addition, 3 out of 17 CDR3's from IgH.ETμ;CD19-hBtk tumors contained stretches of 3 or more tyrosine residues whereas this was only observed in 1 out of 25 tumors from IgH.ETμ and IgH.ETμ;*Btk*$^{+/-}$ mice (data not shown).

These atypical BCR features of IgH.ETμ;CD19-hBtk tumors demonstrate that the increased CLL susceptibility of Btk overexpressing B cells is associated with enhanced transformation of B cells clones expressing non-stereotypic BCRs in IgH.ETμ mice. These BCR characteristics further comprise increased Igλ usage and the presence of long IgH CDR3 regions frequently containing tyrosine stretches, reminiscent of BCRs with self-reactive or poly-reactive specificities.

Discussion

Several recent reports have demonstrated an important role for Btk signaling in the survival and migratory behavior of human CLL cells, but from these studies it was not clear whether Btk signaling is indispensable for CLL development

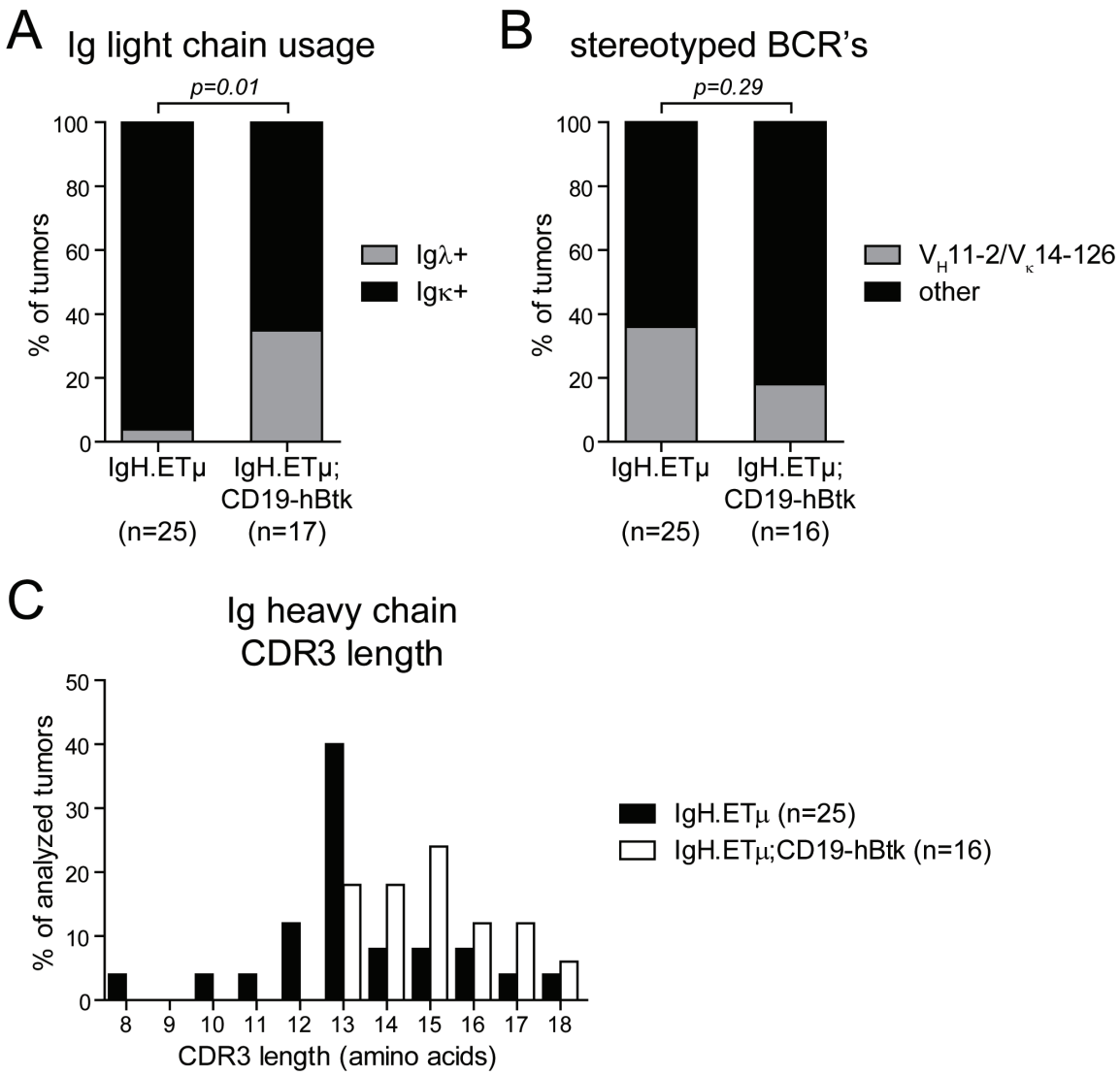

Figure 4. High Btk levels promote atypical BCR clone selection in CLL. A: Proportions of Igλ$^+$ (gray) and Igκ$^+$ (black) CLLs in IgH.ETμ mice (including IgH.ETμ;*Btk*$^{+/-}$ mice; n=25) versus IgH.ETμ;CD19-hBtk mice as determined by flow cytometry (n=16). B: Proportions of stereotypical (V_H11-2$^+$/$V_κ$14-126$^+$) BCR (gray) and non-stereotypical BCR (black) expressing tumors in IgH.ETμ mice (including IgH.ETμ;*Btk*$^{+/-}$ mice; n=25) versus IgH.ETμ;CD19-hBtk mice (n=16). C: Graph summarizing IgH CDR3 length determined by IgH sequencing of tumors from IgH.ETμ mice (including IgH.ETμ;*Btk*$^{+/-}$ mice; black bars, n=25) and IgH.ETμ;CD19-hBtk mice (white bars, n=16).

[16, 17, 20]. Here we show in the IgH.ETμ CLL mouse model that CLL development requires Btk expression: CLL formation was absent in Btk-deficient IgH.ETμ mice, and all tumors derived from Btk haplo-insufficient IgH.ETμ females exclusively expressed the wild-type Btk allele. Although these findings do show that Btk is required for establishing CLL, they do not identify the main mechanism by which Btk promotes CLL development. Given the cardinal role of Btk signaling downstream of the BCR, previous studies initially focused on Btk in BCR signaling in CLL, but now it has become clear that Btk signaling downstream other receptors including CXCR4 and CXCR5 may greatly affect the risk of CLL and of disease progression [16, 17, 20]. This view was affirmed by a transient lymphocytosis, observed in CLL patients and diseased mice alike, upon initiation of treatment with the selective Btk-inhibitor PCI-32765 [17, 32]. It is therefore conceivable that Btk controls CLL formation through multiple pathways including BCR and chemokine receptor signaling, and future research should elucidate

Table 1. Characteristics of stereotypic BCRs in CLLs from IgH.ETμ mice

	tumor	IgH			CDR3	Igκ	
	code	V_H	D_H	J_H		$V_κ$	$J_κ$
IgH.ETμ CLL							
	E01	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	E06	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	E07	11-2	2-1	1	CMRY**G**NYWYFDVW	14-126	4
	E20	11-2	2-1	1	CMRY**G**NYWYFDVW	14-126	2
	E32	11-2	1-1	1	CMRY**GG**YWYFDVW	14-126	2
IgH.ETμ;Btk$^{+/-}$ CLL							
	EH02	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	EH03	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	EH08	11-2	1-1	1	CMRY**GSS**YWYFDVW	14-126	1
	EH10	11-2	2-5	1	CMRYSNYWYFDVW	14-126	1
IgH.ETμ;CD19-hBtk CLL							
	ET08	11-2	2-5	1	CMRYSNYWYFDVW	14-126	2
	ET10	11-2	2-1	1	CMRY**G**NYWYFDVW	14-126	4
	ET16	11-2	2-1	1	CMRY**G**NYWYFDVW	14-126	4

CDR3: complementarity determining region 3. Bold amino acid symbols indicate differences from the CMRYSNYWYFDVW CDR3 motif.

the exact contribution of these Btk signaling pathways to CLL pathogenesis.

By examining the activation of the downstream effector molecules of BCR signaling that are activated to different extents by upstream Btk signals, we tried to identify a Btk signaling signature in BCR-activated CLL cells. Whereas the activation of Akt and Erk upon BCR stimulation was largely unaffected, the activation of Btk's primary signaling target NF-κB was clearly defective in IgH.ETμ tumor cells. These findings are largely in line with earlier characterizations of BCR signaling pathways in CLL that revealed a BCR signaling profile resembling that of anergic or chronically stimulated B cells (reviewed in Kil *et al.* [2]). Nevertheless, it is unlikely that in CLL Btk signaling is completely silenced downstream of the BCR, since it has been shown that PCI-32765 treatment can counteract the pro-survival effects of αIgM stimulation on CLL cells, accompanied by a reduced phosphorylation of Erk and Akt [17]. Our failure to detect induction of NF-κB nuclear translocation in BCR-stimulated CLL cells may therefore be explained by a general retuning or rewiring of BCR signaling pathways in CLL, leading to a shift of Btk signaling targets away from classical targets including NF-κB, which were identified in non-malignant primary B cells. Alternatively, Btk signaling may effectively be weaker in CLL cells compared to non-malignant mature B cells. The possibility that Btk mainly functions as an adaptor protein [24] in signaling complexes in CLL cells is not very likely, because of the reported capacity of PCI-32765 to reduce αIgM-induced phosphorylation of Erk and Akt [16, 17, 20].

The finding that transgenic Btk overexpression (CD19-hBtk) could accelerate and increase CLL formation in IgH.ETμ mice does not directly point to a role for Btk exclusively downstream of the BCR in CLL formation. However, a substantial contribution of Btk-mediated BCR signaling to CLL development is evident from the more frequent occurrence of CLL clones that expressed non-stereotypical BCRs that more frequently employed Igλ and harbored longer CDR3s, demonstrating altered selection based on BCR signaling. This is in line with the finding that, although Btk functions downstream multiple receptors expressed in B cells, increasing Btk expression levels selectively enhances the signaling strength of the BCR, and not of Toll-like receptors or chemokine receptors (Kil et al. [15], and L.P.K. and R.W.H., unpublished). The atypical features of the BCRs found in IgH.ETμ;CD19-hBtk mice also point to a different origin of the B cells that were selected into malignantly transformed CLL clones. It is known that beyond 10 weeks of age, CD19-hBtk mice produce anti-nuclear auto-antibodies, mirroring the presence of auto-reactive or at least poly-reactive cells. Since increased CDR3 length and tyrosine stretches are associated with a higher risk of BCR selfreactivity, it is likely that some of these CLLs expressing atypical

BCRs are derived from auto-reactive or poly-reactive B cells.

The debate on which B cell subsets may contain or represent the precursor cells of CLL is still ongoing, and has led to formulation of hypotheses that these cells may be derived from poly-reactive B1-like cells [33], conventional B2 cells [34], marginal zone B cells [35] or memory B cells [36]. A recent study however, that extensively compared human mature $CD5^+$ B cells to CLL B cells, revealed striking similarities in gene expression profiles and stereotype IgH V gene rearrangements between these two cell populations [3]. Although human $CD5^+$ mature B cells do not represent the exact counterpart of $CD5^+$ mouse B cells which mostly are B1 B cells, this finding could imply that also in murine CLL models (including our IgH.ETμ mice) CLL clones are derived from $CD5^+$ expressing B cells. In this context, the absence of CLL formation in Btk-deficient IgH.ETμ mice could thus result from their lack of B1 B cells [22, 37], rather than from a signaling defect in mature B2 cells. Conversely, enhanced CLL development in CD19-hBtk mice could be related to their ~2-fold increase in B1 B cell numbers [15]. However, it is not likely that the differences in CLL formation can be fully attributed to changes in B1 B cell numbers, since only a minor fraction of the analyzed CLL samples in our panels expressed V_H11 family members, which are vastly overrepresented in B1 B cell populations [38, 39]. We did not observe a proportional increase in V_H11 expressing tumors in CD19-hBtk mice, which would reflect the increase in B1 cell numbers in these mice.

In summary, we have shown that Btk-deficiency fully abrogated CLL formation in mice and that Btk overexpression accelerated CLL onset, increased mortality, and was associated with selection of non-stereotypical BCRs into CLL clones. We therefore conclude that Btk expression levels set the threshold for malignant transformation, further supporting that Btk inhibition by small molecule inhibitors such as PCI-32765 represents an effective new treatment for patients with CLL.

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