PU.1 opposes IL-7-dependent proliferation of developing b cells with involvement of the direct target gene bruton tyrosine kinase

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PU.1 Opposes IL-7–Dependent Proliferation of Developing B Cells with Involvement of the Direct Target Gene Bruton Tyrosine Kinase

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Deletion of genes encoding the E26 transformation-specific transcription factors PU.1 and Spi-B in B cells (CD19-CreΔPB mice) leads to impaired B cell development, followed by B cell acute lymphoblastic leukemia at 100% incidence and with a median survival of 21 wk. However, little is known about the target genes that explain leukemogenesis in these mice. In this study we found that immature B cells were altered in frequency in the bone marrow of preleukemic CD19-CreΔPB mice. Enriched pro–B cells from CD19-CreΔPB mice induced disease upon transplantation, suggesting that these were leukemia-initiating cells. Bone marrow cells from preleukemic CD19-CreΔPB mice had increased responsiveness to IL-7 and could proliferate indefinitely in response to this cytokine. Bruton tyrosine kinase (BTK), a negative regulator of IL-7 signaling, was reduced in preleukemic and leukemic CD19-CreΔPB cells compared with controls. Induction of PU.1 expression in cultured CD19-CreΔPB pro–B cell lines induced Btk expression, followed by reduced STAT5 phosphorylation and early apoptosis. PU.1 and Spi-B regulated Btk directly as shown by chromatin immunoprecipitation analysis. Ectopic expression of BTK was sufficient to induce apoptosis in cultured pro–B cells. In summary, these results suggest that PU.1 and Spi-B activate Btk to oppose IL-7 responsiveness in developing B cells. The Journal of Immunology, 2015, 194: 595–605.

Development of B lymphocytes proceeds through a series of steps marked by Ig gene rearrangement as well as ordered expression of transcription factors (1). Aberrant expression of various transcription factors can predispose developing B cells to leukemia (2). Development of B cell progenitors requires signaling through the IL-7R (3, 4). Productive rearrangement of the IgH locus in pro–B cells permits expression of IgH protein and assembly with surrogate L chain components to promote cell surface expression of a pre-BCR. Signaling through the pre-BCR promotes cell cycle exit and differentiation into small pre–B cells that initiate Ig κ or λ L chain rearrangement (5, 6). Completion of functional Ig L chain rearrangement enables differentiation into B cells expressing a functional BCR.

Several lines of evidence suggest that pre-BCR signaling and IL-7 signaling are mutually antagonistic. IL-7 signaling in pro–B/pre–B cells impairs Igκ rearrangement in a STAT5-dependent manner (7). Pre–B cells are released from the differentiation block induced by IL-7 by moving from microenvironments containing high IL-7 concentrations to low IL-7 concentrations (5, 8). Pre-BCR signaling interferes with the IL-7 signaling pathway using a mechanism that requires B cell linker protein (BLNK/SLP-65) (9). Finally, upon differentiation into immature B cells, the Il7r gene is permanently downregulated at the transcriptional level using mechanisms that are as yet uncharacterized (10). In summary, functional opposition of the pre-BCR and IL-7 signaling pathways is required for differentiation of large pre–B cells into small pre–B cells and Ig L chain rearrangement. Mutations in genes that impair this functional opposition can lead to loss of genomic integrity and leukemia (5).

PU.1 and Spi-B are highly related transcription factors of the E26 transformation–specific family. PU.1 and Spi-B are both expressed in developing B cells and have functional redundancy, likely interacting with a similar set of binding sites in the genome (11, 12). Mutations in SPI1 encoding PU.1 are associated with relapsed B cell acute lymphoblastic leukemia (B-ALL) (13). Repression of the gene encoding Spi-B (Spib) was recently found to be associated with human precursor B-ALL (14) as well as with a mouse model of B-ALL (15). Our laboratory showed that combined null mutation of Spi1 and Spib in mice (CD19 CRE SPI1/Spib−/− mice, called CD19-CreΔPB mice in this study) leads to B-ALL at a median age of 21 wk at 100% incidence (16). The tumor sup-
pressor gene Btk is a direct target of activation by PU.1 and/or Spi-B (17). However, it is not clear how reduced PU.1 and Spi-B predispose developing B cells to malignant transformation in CD19-CreΔPB mice.

Bruton tyrosine kinase (BTK) is a tyrosine kinase that is critically required for pre-BCR/BCR signaling, and when inactivated by mutation, it is the most frequent cause of human X-linked agammaglobulinemia (18, 19). Mutation of Btk in mice is not sufficient to induce leukemia. However, mutation of Btk strongly synergizes with mutation of Blnk (SLP-65) in mice to induce leukemia in mice with ~75% incidence by 16 wk of age (20). Therefore, Blnk and Btk function as cooperating tumor suppressors in developing mouse B cells.

In the present study, we investigated the developmental origins and mechanisms of leukemogenesis in CD19-CreΔPB mice. Bone marrow (BM) pro–B cells were increased in frequency in preleukemic CD19-CreΔPB mice, and they were leukemia-initiating cells as determined by transplantation analysis. Bone marrow cells from preleukemic CD19-CreΔPB mice had increased responsiveness to IL-7 in culture that correlated with loss of BTK expression. Induction of PU.1 expression in cultured CD19-CreΔPB pro–B cells induced Btk expression, reduced STAT5 phosphorylation, and led to early apoptosis. PU.1 and Spi-B regulate PU.1 and Spi-B activate apoptosis in cultured CD19-CreΔPB pro–B cells. Taken together, these results suggest that PU.1 and Spi-B activate Btk to oppose IL-7 responsiveness in developing B cells.

Materials and Methods

Breeding and care of mice

Breeding and genotyping of CD19Cre lox/lox Spi1lox/loxSpi1lox/lox (referred to as CD19-CreΔPB) mice and CD19Cre lox/loxSpi1lox/lox (referred to as ΔB) mice were performed as previously described (16). NOD/SCID/γc–/– (NSG) mice were maintained in a barrier unit at the Western University Animal Care Facility. NSG mice received 300 cGy radiation 2 h before transplantation analysis were performed using the Fix and Perm kit (Life Technologies, Burlington, ON, Canada) and Phosflow PE anti-pSTAT5 pY694 (BD Biosciences). Live/dead cell staining was performed with the Live/Dead fixable yellow dead cell stain kit (Life Technologies). For immunoblotting analysis whole-cell lysates from enriched splenic B cells were probed with anti-BTK Ab (Cell Signaling Technology, Whitby, ON, Canada), HRP goat anti-rabbit secondary Ab (Thermo Fisher), and visualized with SuperSignal West Pico reagent (Thermo Fisher).

Histological and microscopic analysis

Mouse tissue was euthanized by lethal i.p. injection of 540 mg/ml sodium pentobarbital (Euthanyl Forte, Bimeda-MTC Animal Health, Cambridge, ON, Canada). Tissues were fixed in 10% neutral buffered formalin. Femurs were decalcified for 96 h in 26% formic acid (TBD-2, Thermo Fisher, Pittsburgh, PA). Tissues were paraffin embedded, sectioned, and stained with H&E. High-resolution micrographs were captured using a Q-Color3 digital camera (Olympus, Markham, ON, Canada).

Microarray analysis

RNA was prepared from sorted cells using RNA-Beer (Tel-Test, Friends-wood, TX). RNA integrity and purity were checked using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was processed using the WT Terminal Labeling and Hybridization kit (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions and analyzed using Mouse Exon 1.0 ST arrays (Affymetrix) at the London Regional Genomics Centre. Data analysis was performed using Partek Genomics Suite (Partek, St. Louis, MO). Batch effect removal was performed using ComBat software (25). Differentially expressed genes were identified using one-way ANOVA and a cut-off of 1.5-fold with p < 0.05. Heat maps and volcano plots were generated using Hierarchical Clustering Explorer. Partek Pathway software was used to identify biological pathways using the Kyoto Encyclopedia of Genes and Genomes database, and p values were calculated using a Fisher exact test. Microarray data are available from the Gene Expression Omnibus under accession no. GSE48891.

ChIP analysis

Mouse splenic B cells enriched by negative selection of CD43-expressing cells, cultured i660BM cells, or mouse WEHI-279 cells stably expressing 3XFLAG-tagged PU.1 or Spi-B were cross-linked with 1% formaldehyde for 10 min at room temperature. ChIP was performed as previously described (12) using anti-PU.1 (Santa Cruz Biotechnology), anti-FLAG (Sigma-Aldrich), or control mouse anti-IgG Ab (Santa Cruz Biotechnology) conjugated to protein G Dynabeads (Invitrogen, Burlington, ON, Canada). Enrichment was measured using qPCR of DNA immunoprecipitated with anti-PU.1 normalized to anti-IgG, using primers indicated in Supplemental Table I. Fold enrichment was calculated using the comparative threshold cycle method (23). For ChIP sequencing (ChIP-seq) analysis, chromatin solutions with an average DNA fragment size of ~300 bp were generated using a Bioruptor 300 water bath sonicator (Diagenode, South Orange, NJ). DNA was quantified using a 2100 Bioanalyzer (Agilent Technologies). Libraries were generated using the Kapa HT Library Preparation kit (Kapa Biosystems) as per the manufacturer’s recommendations. Size selection was performed on a Pippin Prep instrument (Sage Biosciences). Libraries were quantified using the Quant-IT Picogreen dsDNA assay kit (Life Technologies) and the Kapa Illumina GA with Revised Primers–SYBR Fast Universal kit (Illumina). Average size fragment was determined using a LaChip GX (PerkinElmer) instrument. Libraries were sequenced on an SR100 run on a HiSeq 2000 (Illumina). Two independent ChIP-seq experiments were conducted for PU.1 and Spi-B. Peak finding and data analysis were performed using Galaxy Suite (26). Peaks were called using MACS version 1.0.1 (27) with a genome size of 1.9 × 109 bp (MM9) using input controls. Peaks were called with a tag size set to either 75 (repl1) or 100 (repl2), bandwidth of 300, and a p value cutoff for peak detection of 1 × 10−5. Peak Model was generated using an in silico high-confidence enrichment ratio against background of 15. Wiggle files were created using a resolution of 10 bp. Independent experiments were combined and normalized to call consensus peaks using mmChIP v1.0.0 with a mappable genome size of 191 × 109 bp and a p value cutoff for peak detection of 1 × 10−5 (28). Input bed
files for mmChip were generated using Convert from BAM to BED tool v0.1.0 in Galaxy. ChiP-sequence data are available from the Gene Expression Omnibus under accession no. GSE58128.

**Statistical analysis**

All data are reported as means ± SD. Statistical significance was determined using a Student t test on biological replicates unless otherwise indicated. Values with p < 0.05 were considered significant. Statistical analysis was performed using Prism 5.0 (Graphpad Software, La Jolla, CA).

**Results**

**Increase in frequency of immature B cells in the BM of CD19-CreΔPB mice**

Deletion of genes encoding PU.1 and Spi-B in B cells (CD19-CreΔPB mice) leads to impaired B cell development, followed by B-ALL at 100% incidence and with a median survival of 21 wk (16). However, the developmental origin of B-ALL in CD19-CreΔPB mice was not previously examined. BM B cell development was examined in detail using flow cytometry and the “Hardy” classification system of fractions A–F (29). Both wild-type (WT; C57BL/6) and ΔB (Spib−/−) BM cells were included as controls because these two types of mice do not develop leukemia.

First, BM cells were prepared from 6- to 10-wk-old WT, ΔB, or CD19-CreΔPB mice. Total B220+ BM cell frequencies were slightly increased in 6- to 10-wk-old ΔB mice but were decreased in 6- to 10-wk-old CD19-CreΔPB mice (Fig. 1A, 1C). Frequencies of B220+CD43− IgM+ cells (fraction E immature B cells) were significantly increased in 6- to 10-wk-old ΔB and CD19-CreΔPB mice, whereas frequencies of B220+CD43− IgM- (fraction F mature recirculating B cells) were significantly decreased (Fig. 1A, 1D). Analysis of BM development was also performed in mice aged 11–18 wk. Total B220+ BM cell frequencies were dramatically decreased in 11- to 18-wk-old CD19-CreΔPB mice (Fig. 1E). Frequencies of fraction C pro–B cells and fraction E immature B cells were dramatically expanded, representing nearly 30 and 50%, respectively, of all B220+ cells in older CD19-CreΔPB mice (Fig. 1B, 1F). Frequencies of fraction F B cells were reduced even further in older mice, as previously reported for mature splenic B cells (16) (Fig. 1B, 1F). In conclusion, immature B cells were increased in frequency in the BM of adult CD19-CreΔPB mice preceding overt leukemia.

**Enriched fraction C pro–B cells from BM of CD19-CreΔPB mice transfer disease**

Increased frequencies of fraction C pro–B cells in CD19-CreΔPB mice suggested that these might represent leukemia-initiating...
cells. To test this idea, transplantation experiments were performed using sorted cells from 6- to 10-wk-old ΔB (control) or CD19-CreΔPB mice. First, mature splenic B cells (B220<sup>+</sup>CD19<sup>+</sup>CD93<sup>+</sup>Ig<sup>k</sup> cells) were enriched using cell sorting and injected i.v. into immunodeficient NSG mice (30). Recipient mice receiving 5 × 10<sup>5</sup> mature splenic B cells of either genotype showed no signs of illness until the experiment was terminated at 4 mo (Fig. 2A). Next, NSG mice were transplanted with 10<sup>5</sup> fraction C pro–B cells from ΔB or CD19-CreΔPB donors. Fraction C pro–B cells were enriched by cell sorting using the gating strategy shown in Fig. 1A. NSG mice injected with ΔB pro–B cells did not show signs of disease at any time point (Fig. 2B). In contrast, NSG mice receiving CD19-CreΔPB fraction C pro–B cells became ill and required euthanasia with a median time of 113.5 d (Fig. 2B). The spleens of NSG mice receiving CD19-CreΔPB fraction C pro–B cells contained high frequencies of leukemia cells expressing BP-1 and CD93 as determined by flow cytometry (Fig. 2C). Histopathological examination revealed that every tissue examined in NSG mice receiving CD19-CreΔPB pro–B cells was infiltrated with leukemia cells. Spleen and BM of transplanted NSG mice showed extensive infiltration of leukemia cells resulting in effacement of the normal architecture (Fig. 3A, 3B). Liver showed extensive infiltration of leukemia cells throughout the sinusoids with focal aggregates of leukemia cells in the portal and periportal regions (Fig. 3B, Supplemental Fig. 1A). The cortex of the brain showed meningeal invasion of leukemia cells (Fig. 3D, Supplemental Fig. 1B) whereas pancreatic tissues showed a large metastatic aggregate of leukemia cells (Fig. 3E). In conclusion, these results suggest that BM fraction C pro–B cells are leukemia-initiating cells in CD19-CreΔPB mice.

Analysis of gene expression in leukemic cells from CD19-CreΔPB mice reveals a pattern of impaired BCR signaling

Analysis of gene expression can provide important insights into pathways of oncogenic transformation (31). As a control for PU.1-dependent gene expression, cell sorting was used to enrich splenic CD19<sup>B220<sup>+</sup> cells from the spleen of ΔB mice (Fig. 4A, left panel). For the experiment, cell sorting was used to enrich splenic CD19<sup>B220<sup>+</sup> B-ALL cells or residual CD19<sup>B220<sup>+</sup> B cells from leukemic CD19-CreΔPB mice (Fig. 4A, right panel). RNA was prepared from sorted samples from these three groups and analyzed for gene expression using Affymetrix 1.0 ST exon arrays. Using a cutoff of a 1.5-fold change and p value of <0.05 by one-way ANOVA, 2908 genes were found to be downregulated and 3477 genes were upregulated in CD19-CreΔPB CD19<sup>B220<sup>low</sup>-ALL cells compared with ΔB control cells. For the top 100 differentially expressed genes, downregulated genes were reduced by an average of 56-fold and upregulated genes increased by an average of 27-fold (Fig. 4B). Exon-specific analysis confirmed that the 3′ end of the Spi1 transcript encoding PU.1 was reduced by an average of 20-fold in CD19-CreΔPB B-ALL cells, whereas the 5′ end was transcribed equivalently, as predicted by the Cre-mediated deletion of exons 4 and 5 in these mice (16). Gene expression was also compared with residual CD19<sup>B220<sup>+</sup> B cells from CD19-CreΔPB mice. Interestingly, these cells showed changes in gene expression that were intermediate between CD19-CreΔPB CD19<sup>B220<sup>low</sup>-ALL cells and ΔB B cells (Fig. 4C). CD19-CreΔPB B cells expressed Spi1 transcripts at levels intermediate between the two other populations, suggesting that these were B cells with delayed deletion of Spi1 (Fig. 4C).

In the published literature at least 110 genes have been shown to be directly regulated by PU.1 using biochemical approaches (32). Of these 110 genes, 44 (40%) were downregulated in CD19-CreΔPB B-ALL cells compared with controls and 11 (10%) were upregulated. The remaining 55 genes (50%) were not significantly altered. Thus, 50% of known PU.1 target genes in this list were significantly altered. For genes that were upregulated in CD19-CreΔPB B-ALL cells compared with control ΔB B cells, the top two pathways as ranked by enrichment score and p value included cell cycle and DNA replication. Specific genes that were highly expressed in CD19-CreΔPB B-ALL cells and were confirmed by RT-qPCR included Il7r (encoding IL-7Rα), Lgr5, Lef1, Enpep...
genes that were downregulated in CD19-CreΔPB B-ALL cells compared with control ΔB B cells, the biological pathway with the lowest p value identified using gene ontology analysis was BCR signaling. A large number of genes in this biological pathway were significantly downregulated, including Shp1, Syk, Lyn, Btk, Blnk, Vav1, Rac, Bcap, and Grb2. Several of these downregulated genes were confirmed by RT-qPCR analysis of three biological replicates (Fig. 4E). This analysis confirmed downregulation of Btk (encoding B cell linker protein), Lyn (encoding the tyrosine kinase Lyn), and nearly 100-fold downregulation of Blnk (encoding BP-1), and Tcfl2 (Fig. 4B, 4E). As expected based on differential B220 staining, Ptprc (encoding B220) was one of the most highly downregulated genes (Fig. 4B, 4E). Importantly, for

Altered IL-7 responses in developing B cells from CD19-CreΔPB mice

IL-7Rαx was among the most highly increased genes in our analysis (Fig. 4C, 4E). To determine whether immature B cells in the BM of preleukemic CD19-CreΔPB mice had altered responses to IL-7, unfractionated BM cells from 6- to 8-wk-old WT, ΔB, or CD19-CreΔPB mice were placed in culture with ST2 stromal cells and IL-7. Cell counts and passages were performed every 4 d until proliferation ceased. BM cells from ΔB and CD19-CreΔPB mice reproducibly generated more proliferating cells than did WT mice upon initial culture (representative experiment shown in Fig. 5A). Interestingly, ΔB and CD19-CreΔPB BM cells contained higher levels of phosphorylated STAT5 upon IL-7 restimulation than did WT BM cells (Supplemental Fig. 2). However, WT and ΔB cultures did not continue to proliferate after three passages. In contrast, BM cells from CD19-CreΔPB mice proliferated indefinitely and in all five trials could be readily established as cell lines (Fig. 5A). These results suggested that BM pro–B cells from CD19-CreΔPB mice had increased IL-7 sensitivity.

Mutation of Btk in mice has previously been shown to lead to increased IL-7 sensitivity in mouse BM cells (20, 33). Therefore, we determined whether Btk mRNA transcript levels correlated with Spi1 mRNA transcript levels during the process of Spi1 deletion under the control of CD19-Cre in cultured CD19-CreΔPB BM cells. RT-qPCR analysis was performed on RNA prepared from CD19-CreΔPB BM cells placed in culture with IL-7 and passaged up to eight times. At early passage numbers, cultured CD19-CreΔPB pro–B cells expressed high levels of B220 (Fig. 5B, left panels), Spi1 transcripts (Fig. 5C), and Btk transcripts (Fig. 5E). After repeated passage, expression of B220, Spi1, and Btk were lost (Fig. 5B, right panels, 5C–E). IL7r transcripts increased on a per cell basis from passage 1 to passage 8 (Fig. 5D). Cell lines established from the BM of 6-wk-old CD19-CreΔPB mice expressed IL-7Rαx, c-Kit, and CD19 (Fig. 5B, 5E). Proliferation of the cells was IL-7–dependent. Two of five cell lines had detectable Igh rearrangements (Fig. 5G). However, these cell lines did not express Igh on the cell surface. None of the five lines established had detectable Igk rearrangements, or expressed Igk protein on the cell surface. Therefore these cell lines resembled cultured pro–B cells rather than pre–B cells. In conclusion, the changes that occurred in cultured CD19-CreΔPB BM cells during the process of Spi1 deletion, including increased IL-7Rαx expression, reduced B220 expression, and reduced Btk expression, were similar to differences between ΔB B cells and CD19-CreΔPB leukemia cells (Fig. 4). These changes might therefore occur in the BM of CD19-CreΔPB mice during the process of Spi1 deletion and precede leukemic transformation.
PU.1 induces Btk expression and loss of proliferation in cultured CD19-CreΔPB pro–B cells

To determine the consequences of restoring PU.1 expression in CD19-CreΔPB cells, we made use of a doxycycline-inducible system (22). A BM-derived CD19-CreΔPB pro–B cell line, 660BM, was generated as described in Fig. 5. 660BM cells were IL-7–dependent and rapidly died by apoptosis when deprived of IL-7. 660BM cells were infected with a retroviral regulator vector encoding Tet3G and a retroviral response vector encoding PU.1 under the control of a Tet3G-responsive promoter (Fig. 6A). A clonal cell line termed i660BM was generated by picking a single GFP+ colony grown in methylcellulose containing IL-7 and puromycin. Spi1 (encoding PU.1) expression could be highly induced in i660BM cells with concentrations of doxycycline as low as 70 ng/ml (Fig. 6B). Interestingly, 48 h after PU.1 induction, i660BM cells shrunk in size as determined by reduced forward light scatter (Fig. 6C). Transition of high forward scatter to low forward scatter cells was doxycycline dose–dependent (Fig. 6D). All low forward scatter cells stained brightly with annexin V, indicating early apoptosis, whereas high forward scatter cells had low levels of annexin V staining (Fig. 6E). Induction of PU.1 in i660BM cells resulted in reduced STAT5 phosphorylation, suggesting that signaling downstream of the IL-7R was inhibited in these cells (Fig. 6F). In summary, these results show that induction of PU.1 reduced STAT5 phosphorylation, reduced cell size, and induced early apoptosis.

PU.1 and Spi-B directly regulate the Btk gene

The promoter region of Btk contains binding sites capable of interacting with PU.1 (34, 35). To determine whether PU.1 can induce BTK expression, we performed anti-BTK immunoblot analysis. Inducible i660BM cells did not express detectable BTK

**FIGURE 4.** Gene expression analysis of CD19-CreΔPB leukemia suggests impaired BCR signaling. (A) Sort gates for splenic ΔB B cells and CD19-CreΔPB B-ALL cells. Three ΔB B cell samples, three CD19-CreΔPB B220CD19− B-ALL samples, and two CD19-CreΔPB B220CD19− residual B cell samples were collected and analyzed individually by microarray. (B) Volcano plot of changes in gene expression in splenic CD19-CreΔPB B-ALL cells compared with ΔB B cells. Genes of interest are indicated by arrowheads. (C) Heat map comparing changes in gene expression in splenic CD19-CreΔPB B-ALL cells (ΔB B-ALL), splenic CD19-CreΔPB B220 B cells (ΔB B), and ΔB B cells (ΔB B). The top 50 decreased genes are shown on the left; the top 50 increased genes are shown on the right. (D) Venn diagram showing overlap between published PU.1 target genes and genes downregulated in CD19-CreΔPB B-ALL cells compared with ΔB B cells. Of 110 published PU.1 target genes, 44 were significantly downregulated in CD19-CreΔPB B-ALL cells. (E) Confirmation of alternatively expressed mRNA transcripts. RT-qPCR was used to confirm relative upregulation of five indicated genes and downregulation of four indicated genes in CD19-CreΔPB B-ALL cells compared with ΔB B cells. Error bars represent means and SD of three biological replicates and all had p values ≤ 0.05 by the Student t test.
protein when cultured without doxycycline. However, BTK was expressed after PU.1 induction (Fig. 7A). RT-qPCR measurement of gene expression showed that Btk mRNA transcripts were highly induced by PU.1 (Fig. 7B). To determine whether PU.1 directly regulates Btk in B cells, we performed anti-PU.1 ChIP experiments. In i660BM cells, interaction of PU.1 with the Btk promoter was inducible by doxycycline at 24 (Fig. 7C) and 48 h (Fig. 7D). In mouse splenic B cells, PU.1 was also found to interact with the Btk promoter as well as with the Mef2c enhancer as a positive control (Fig. 7E). Therefore, PU.1 was found to interact with the Btk promoter in both pro-B-like cells and mature B cells. For mouse Spi-B, no ChIP-quality Abs are currently available. Therefore, to determine whether Spi-B could also interact with the Btk promoter, we performed ChIP-seq analysis using anti-FLAG ChIP in 3× FLAG–tagged PU.1 or Spi-B–infected mouse WEHI-279 cells (L.A. Solomon, S.K.H. Li, J. Piskorz, L.S. Xu, and R.P. DeKoter, submitted for publication). Analysis of ChIP-seq data identified nearly identical peaks of PU.1 or Spi-B interaction near the annotated transcription start site of Btk (Fig. 7F). These data therefore indicate that Btk may be regulated by both PU.1 and Spi-B. To determine whether Btk expression requires PU.1 in vivo, we examined BTK protein levels by immunoblot of the few B cells that are present in the spleen of preleukemic CD19-CreΔPB mice. We found that BTK protein levels were reduced in CD19-CreΔPB B cells compared with either WT or ΔB B cells (Fig. 7G). Collectively, these results show that PU.1 and Spi-B can activate BTK expression in the B cell lineage. Restoration of BTK expression was previously shown to induce apoptosis in cultured leukemia cells lacking BTK expression (36). To determine whether BTK expression is sufficient to induce apoptosis in cultured 660BM cells, these cells were infected with pMSCV-hBTK-FLAG retrovirus encoding BTK and GFP (37) or pMSCV-ires-GFP alone as a control. Infection of 660BM cells with BTK induced early apoptosis by 48 h as determined by annexin V staining (Fig. 7H, 7I). We conclude that ectopic expression of BTK in 660BM cells is sufficient to induce early apoptosis.
Discussion

In the present study, we showed that deletion of the genes encoding PU.1 and Spi-B leads to an alteration of immature B cell frequencies in the BM. Enriched pro–B cells from 6- to 10-wk-old CD19-CreΔPB mice induced leukemia in transplanted recipients, suggesting that these were leukemia-initiating cells. Analysis of gene expression in B-ALL cells from CD19-CreΔPB mice showed that PU.1 and Spi-B enforce expression of many genes required for BCR signaling, including Btk. PU.1 was sufficient to induce Btk expression in a CD19-CreΔPB pro–B cell line. ChIP experiments demonstrate that Btk is directly activated by PU.1 and Spi-B. BTK expression was dramatically reduced in splenic B cells lacking PU.1 and Spi-B. Ectopic expression of BTK was sufficient to induce apoptosis in cultured pro–B cells lacking PU.1 and Spi-B. Taken together, these results show that PU.1 and Spi-B are required for BTK expression in B cells, and reduced BTK expression as a consequence of PU.1/Spi-B deletion is associated with abnormal IL-7–dependent expansion of pro–B cells in the BM.

The present study showed that a key gene dramatically downregulated in B-ALL cells from CD19-CreΔPB mice was Btk (Fig. 6). Btk was previously implicated as a potential target gene of PU.1 (34, 35), a result that is now confirmed in this study. Our experiments demonstrated that PU.1 interacts directly with the Btk promoter in WEHI-279 cells and the pro–B cell line 660BM, and that Spi-B interacts with the Btk promoter in WEHI-279 cells. It remains to be determined whether Spi-B interacts with the Btk
promoter in pro–B cells. Btk gene expression was directly correlated with Spi1 transcript levels in cultured CD19-CreΔPB BM cells during the process of Spi1 deletion (Fig. 5C, 5E) consistent with the idea of this gene being PU.1-dependent. In contrast, Btk transcript levels inversely correlated with Il7r transcript levels (Figs. 4E, 5D) as well as with IL-7 responsiveness (Fig. 5A). Deletion of the Btk gene was previously shown to be sufficient to cause increased IL-7 responsiveness in BM pre–B cells (20). BTK is a key component of the pre-BCR signaling pathway that functions to block IL-7R signaling in developing B cells (38). We note that the 660BM cells used in our study resemble pro–B cells rather than pre–B cells based on their lack of expression of surface IgM or Igk. Therefore, BTK may function to oppose IL-7R signaling in pro–B cells that do not yet express a pre-BCR or BCR. In summary, reduced BTK expression caused by deletion of PU.1 and/or Spi-B may be sufficient to explain why CD19-CreΔPB pro–B cells are hyperresponsive to IL-7 in culture. Increased responsiveness of CD19-CreΔPB pro–B/pre–B cells to IL-7 may also explain why fraction C pro–B cells expand in the BM of preleukemic mice.

Our laboratory previously showed that Blink (encoding B cell linker protein) is a direct target of PU.1 and Spi-B in developing B cells (17). Loss-of-function mutation of Btk strongly synergizes with loss-of-function mutation of Blink in mice to induce B-ALL in mice with ~75% incidence by 16 wk of age (20). BM cells from Btk/Blink knockout mice have increased sensitivity to IL-7 and increased ability to proliferate in response to IL-7 (33, 39, 40). Similar to CD19-CreΔPB mice, leukemias from Btk/Blink knock-
out mouse express BP-1, CD43, and IL-7R and can be readily cultured as cell lines in response to IL-7 (9, 20). Taken together, these similarities suggest that reduced expression of both BLNK and BTK, as a consequence of PU.1 and Spi-B deletion, may explain why developing CD19-CreΔPB B cells are susceptible to malignant transformation. It is still not fully understood why reduced BTK and BLNK lead to leukemia in mice. It has been speculated that the mechanism might involve prolonged transit through the V(D)J recombination process, leading to accumulation of undesired mutations (41). Expression of an Igh transgene prevails in Btk/Blnk knockout mice, possibly because this accelerates transition through Ig recombination (41). We note that B-ALL cells from CD19-CreΔPB mice express high levels of RAG-1 and RAG-2 as determined by microarray analysis (11- and 9-fold higher than control cells, respectively). More investigation will be needed to determine whether ongoing Ig recombination in CD19-CreΔPB B cells plays a role in leukemic transformation.

To summarize, we propose a model to explain why deletion of PU.1 and Spi-B in the B cell lineage leads to impaired B cell development and eventually B-ALL. In CD19-CreΔPB mice, the Spi1 gene is deleted starting at the pro–B cell stage under the control of CD19. Pro–B cells that delete Spi1 downregulate expression of the target genes Btk and Blnk. Reduced BTK and BLNK expression would in turn result in increased IL-7R sensitivity, leading to increased proliferation of pro–B and large pre–B cells. Additionally, reduced BTK and BLNK might lead to delayed V(D)J recombination, leading to accumulation of mutations. Most cells that delete Spi1 likely do not progress past the pre–B cell stage due to impaired BTK/BLNK-dependent pre-B cell signaling. We speculate that leukemia arises in rare progenitor cells from the late pro–B/early pre–B fraction C and C′ compartments.

There are a number of similarities between the CD19-CreΔPB mouse model of B-ALL and human precursor B-ALL. Mutations in SPI1 encoding human PU.1 have been identified in relapsed precursor B-ALL (13), and reexpression of SPI1B encoding human Spi-B is associated with human precursor B-ALL (14). Btk and Blnk were found to be targets of PU.1 and Spi-B in our studies, and they are established as tumor suppressors in human precursor B-ALL (36, 42). Expression of the IL-7R has long been known to be a common feature of human childhood or adult B cell leukemia (43, 44). Finally, although IL-7 has been previously suggested to be not required for human B cell development, gain-of-function mutations in the human IL7R gene are associated with precursor B-ALL (45, 46). These correlations lead us to believe that further studies using the CD19-CreΔPB mouse model will lead to elucidation of the biochemical pathways that may explain how precursor B-ALL arises in human patients.

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Disclosures

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References


