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10-1-2012

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The Journal *of* Immunology

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Regulation of B Cell Linker Protein Transcription by PU.1 and Spi-B in Murine B Cell Acute Lymphoblastic Leukemia

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B cell acute lymphoblastic leukemia (B-ALL) is frequently associated with mutations or chromosomal translocations of genes encoding transcription factors. Conditional deletion of genes encoding the E26-transformation–specific transcription factors, PU.1 and Spi-B, in B cells (ΔPB mice) leads to B-ALL in mice at 100% incidence rate and with a median survival of 21 wk. We hypothesized that PU.1 and Spi-B may redundantly activate transcription of genes encoding tumor suppressors in the B cell lineage. Characterization of aging ΔPB mice showed that leukemia cells expressing IL-7R were found in enlarged thymuses. IL-7R–expressing B-ALL cells grew in culture in response to IL-7 and could be maintained as cell lines. Cultured ΔPB cells expressed reduced levels of B cell linker protein (BLNK), a known tumor suppressor gene, compared with controls. The *Blnk* promoter contained a predicted PU.1 and/or Spi-B binding site that was required for promoter activity and occupied by PU.1 and/or Spi-B as determined by chromatin immunoprecipitation. Restoration of BLNK expression in cultured ΔPB cells opposed IL-7–dependent proliferation and induced early apoptosis. We conclude that the tumor suppressor BLNK is a target of transcriptional activation by PU.1 and Spi-B in the B cell lineage.   *The Journal of Immunology*, 2012, 189: 3347–3354.

A cute lymphoblastic leukemia (ALL) is the most common form of cancer in young children (1). Despite a better than 80% cure rate, ALL represents a leading cause of leukemia-related deaths in children and upon relapse in adults (1). The majority of human ALLs are cancers of the B lymphocyte lineage (B cell ALL [B-ALL]) and are frequently associated with mutations or chromosomal translocations targeting genes encoding transcription factors (2). For example, 25% of cases of pre-B ALL have the t(12;21) chromosomal translocation, resulting in a fusion between the *ETV6* and *RUNX1* genes encoding the transcription factors TEL and AML1, respectively (1). The E26-transformation–specific transcription factor PU.1, encoded by the gene *Sfpi1* in mice and *SPI1* in humans, is required for generating lymphoid progenitor cells and is a key regulator of B cell fate specification (3, 4). *SPI1* mutations are associated with human AML (5, 6) and B-ALL (7). Reduced PU.1 expression is sufficient to induce AML in mice (8, 9). Reduced PU.1 levels are also associated with human lymphoid leukemia and lymphoma (10, 11).

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Spi-B (encoded by *Spib*) is expressed in developing B cells (12), and it interacts with DNA binding sites thought to be identical to those recognized by PU.1 (13). Reduced Spi-B expression was recently associated with human B-ALL (14). Our laboratory previously showed that mice that lack both PU.1 and Spi-B in the B cell lineage (*CD19*[+/Cre]*Sfpi1*[lox/lox]*Spib*^{−/−} mice, henceforth known as ΔPB mice) have impaired B cell development and develop B-ALL with 100% incidence rate by 21 wk of age (15). These results demonstrate that PU.1 and Spi-B have tumor-suppressor function in the B cell lineage and suggest that mutation of the *SPI1* and *SPIB* genes, or upstream activators of these genes, could be oncogenic drivers in leukemia-initiating cells. Because these proteins are transcription factors, it is important to identify target genes that could explain the role of PU.1 and Spi-B in lymphoid leukemogenesis.

B cell linker protein (BLNK, also known as SLP65 or BASH) is an adaptor protein that is required for B cell development as a consequence of its important role in BCR signaling (16–18). BLNK links BCR signaling with enforcement of B cell differentiation by mediating interactions between Syk, Vav, PLCγ2, Grb2, and Bruton's tyrosine kinase (Btk) upon phosphorylation by Syk (19, 20). Inactivating mutations of *BLNK* are associated with B-ALL in human patients (21, 22), and reduced expression of BLNK has also been associated with B-ALL (23). In mice, mutation of *Blnk* is sufficient to induce B-ALL with a 10% incidence rate (23, 24). Therefore, BLNK has important roles in enforcing B cell differentiation and functions as a tumor-suppressor gene.

We previously noted that levels of *Blnk* mRNA transcripts were reduced in sorted splenic ΔPB B-ALL cells compared with control B cells (15). Therefore, we hypothesized that *Blnk* is a target of transcriptional activation by PU.1 and/or Spi-B. To test this hypothesis, we performed a detailed characterization of B-ALL cells from ΔPB mice. ΔPB B-ALL cells expressed high levels of IL-7Rα and grew in culture in response to IL-7. Cultured ΔPB B-ALL cells expressed reduced levels of BLNK mRNA transcripts and protein compared with control cells. The *Blnk* promoter was

confirmed as a direct target of activation by PU.1 and/or Spi-B using transient transfection and chromatin immunoprecipitation (ChIP) analysis. Finally, we showed that forced expression of BLNK in cultured ΔPB B-ALL cells opposed proliferation by inducing early apoptosis. We conclude that the tumor suppressor BLNK is a target of transcriptional activation by PU.1 and Spi-B in the B cell lineage.

Materials and Methods

Breeding and care of mice

Mice used in this study were on the C57BL/6 background and were generated by mating $CD19^{+/Cre}Sfpi1^{lox/lox}Spib^{-/-}$ (ΔPB) males to $CD19^{+/+}$ $Sfpi1^{lox/lox}Spib^{-/-}$ (ΔB) females, and genotyped as previously described (15). Mouse care was monitored under an approved animal use protocol in accord with the University of Western Ontario Council on Animal Care.

Cell culture

ΔPB B-ALL cells were cultured in IMDM (Lonza, Shawinigan, QC, Canada) containing 5% IL-7–containing conditioned media from the J558L–IL-7 cell line (25), 10% FBS (Biologos, Montgomery, IL), 1× penicillin/streptomycin/L-glutamine (Lonza, Shawinigan, Quebec, Canada), and 5×10^{-5} M 2-ME (Sigma-Aldrich, St. Louis, MO). Purified recombinant growth factors were purchased from PeproTech (Embrun, ON, Canada) and used at the following concentrations: murine stem cell factor (100 ng/ml), murine M-CSF (10 ng/ml), murine IL-3 (10 ng/ml), murine IL-6 (10 ng/ml), murine GM-CSF (1 ng/ml), murine Flt-3 ligand (20 ng/ml), murine IL-7 (100 ng/ml), and murine G-CSF (10 ng/ml). WEHI-279 B cell lymphoma cells were maintained in complete DMEM (Lonza) containing 4.5 g/l glucose. ST2 stromal cells were maintained in complete IMDM. Plat-E retroviral packaging cells and NIH-3T3 cells were maintained in complete DMEM containing 1.5 g/l glucose. All cell lines were maintained in 5% CO_2 atmosphere and at 37°C.

Colony-forming assays

ΔPB cells or retrovirally infected ΔPB cells were plated in methylcellulose media containing recombinant murine IL-7 (MethoCult 03630; Stem Cell Technologies, Vancouver, BC, Canada) and incubated for 7 d at 37°C and an atmosphere of 5% CO_2. Colonies were scored and visualized using a Zeiss A-plan 5×/0.12 Pho 441021-9910 objective lens on an Axio Observer A1 microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Flow cytometric analysis and immunoblotting

Abs used in this study were purchased from eBioscience (San Diego, CA) and included biotin anti–pre-BCR (clone SL156), biotin anti-IgM (II/41), PE anti–c-Kit/CD117 (2B8), biotin anti-CD45R/B220 (2A3-6B2), biotin anti–IL-7Rα/CD127 (B12-1), PE-conjugated anti-CD93 (AA4.1), FITC-conjugated anti-BP-1 (6C3), and PE-, allophycocyanin-, or Cy5-conjugated streptavidin as secondary reagents. Alexa Fluor 647-Annexin V was purchased from BioLegend (San Diego, CA). Flow cytometric analysis was performed using a BD FACSCalibur system (BD Biosciences, Franklin Lakes, NJ). Data analysis was performed using FlowJo 9.4.10 Software (Tree Star, Ashland, OR). All analyses shown are gated on a viable lymphocyte population based on forward and side scatter values. Immunoblotting was performed using standard methods or as previously published (26). Abs used included rabbit anti-Akt, rabbit anti–phospho-Akt, rabbit anti-BLNK (Cell Signaling Technology, Beverly, MA), rabbit anti-PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti–β-actin peptide Ab (Santa Cruz), HRP-conjugated anti-rabbit or anti-goat secondary Abs (Pierce Biotechnology, Nepean, ON, Canada), and Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Bioinformatics analysis

Phylogenetic comparison of the upstream region of the *Blnk* gene from human and mouse was performed using MacVector 11.0 (Accelrys, San Diego, CA) using sequences obtained from the Ensembl.org database. Identification of potential E26-transformation–specific binding sites within the upstream region of the *Blnk* gene was performed using the application MATINSPECTOR (Genomatix, Munich, Germany) (27).

Plasmid construction

A 405-bp region upstream of the translation start site in the mouse *Blnk* promoter was amplified by PCR using LA-TAQ (TaKaRa; Fisher Scientific, Toronto, ON, Canada), and 5′ and 3′ HindIII recognition sites were

added to the PCR primers. The PCR fragment was cloned using the StrataClone PCR Cloning Kit (Agilent Technologies Canada, Mississauga, ON, Canada). A HindIII fragment containing the *Blnk* promoter was ligated in forward and reverse orientation into the HindIII site of pGL3-basic (Promega, Madison, WI). To obtain pGL3-*Blnk*-MUT plasmid, we subjected the pGL3-*Blnk*-FWD to site-directed mutagenesis using the QuikChange Lightening Site-Directed Mutagenesis Kit (Agilent). *Blnk* cDNA was generated from RNA isolated from spleen of a C57B1/6 mouse using the RNA-Bee isolation reagent (Tel-Test, Friendswood, TX) and reverse transcribed using the iScript kit (Bio-Rad Canada, Mississauga, ON, Canada). The 1501-bp *Blnk* cDNA was amplified by PCR using LA-TAQ (TaKaRa) and cloned using the StrataClone PCR Cloning Kit (Agilent). An EcoR1 fragment containing the *Blnk* cDNA was ligated into the EcoR1 site of MIGR1. To generate MIG-3XFLAG-PU.1 and MIG–3XFLAG–Spi-B, we used PCR to amplify the 3XFLAG coding sequence from the plasmid vector pBICEP–CMV-2 (Sigma-Aldrich) to introduce a 5′ XhoI site and retain an in-frame NotI site. This DNA fragment was cloned and ligated into the retroviral vectors MIG-HA-PU.1 and MIG–HA–Spi-B (28) using XhoI and NotI sites to replace the hemagglutinin epitope tag sequence of this vector. All constructs were confirmed by DNA sequencing. Oligonucleotide sequences are listed in Supplemental Table I.

Retroviral production and infection

MIGR1, MIG-3XFLAG-PU.1, MIG–3XFLAG–Spi-B, and MIG-BLNK retroviruses were generated by transient transfection of Plat-E packaging cells (29) using polyethylenimine transfection at a 3:1 polyethylenimine/DNA ratio (30). Virus-containing supernatants were collected at 48 h posttransfection, and viral titers were measured by infection of NIH-3T3 cells. Infections were performed by "spinoculation" with high-titer virus by centrifugation at $2000 \times g$ for 3 h at 32°C in the presence of 8 μg/ml polybrene. After centrifugation, cells were washed and cultured for 48 h to promote retroviral integration and gene expression. Infection frequencies were detected by flow cytometric analysis of GFP.

Transient transfection and ChIP analysis

Transient transfection of WEHI-279 cells was performed using electroporation as previously described (31). MIGR1, MIG-3XFLAG-PU.1, and MIG–3XFLAG–Spi-B–infected WEHI-279 clonal cell lines were cross-linked with 1% formaldehyde for 10 min at room temperature. ChIP was performed as previously described (31). Enrichment was measured using quantitative PCR (qPCR) of DNA immunoprecipitated with anti-FLAG magnetic beads (Sigma-Aldrich), using primers indicated in Supplemental Table I. Percentage of input was calculated using the comparative threshold cycle method (32).

Statistical analysis

All data are reported as mean ± SD of the mean. Statistical significance was determined using a Student t test unless otherwise indicated. The p values <0.05 were considered significant. Statistical analysis was performed using Prism 5.0 (GraphPad Software, La Jolla, CA).

Results

Leukemia cells in the thymus of ΔPB mice express IL-7Rα

Mice that lack PU.1 and Spi-B in the B cell lineage (ΔPB mice) require euthanasia at a median age of 21 wk as a consequence of lethal B-ALL (15). Leukemia cells infiltrate all lymphoid tissues in these mice, but the proximal cause of death is dyspnea associated with an enlarged thymus (15). To determine whether thymus enlargement is age dependent, groups of ΔPB mice were analyzed at various ages between 6 and 23 wk. Thymuses were never enlarged in mice aged 6–10 wk (0/8). However, thymuses were enlarged in 6 of 9 mice aged 11–18 wk and 11 of 12 mice aged 19–23 wk (Fig. 1A). The weight of enlarged thymuses in 19- to 23-wk-old ΔPB mice was >10-fold greater than that of littermates that lack only Spi-B (ΔB; Fig. 1B). To confirm the identity of cells in enlarged thymuses of ΔPB mice, we performed flow cytometric analysis. Every enlarged thymus, regardless of age, contained >90% CD19$^+$ cells, indicating that they were of the B cell lineage (Fig. 1D). All CD19$^+$ B-ALL cells also expressed cell-surface IL-7Rα (CD127; Fig. 1C, 1D). The one thymus that was not enlarged in the 19- to 23-wk age group (Fig. 1A, *right*

FIGURE 1. Age-dependent appearance of IL-7Rα–expressing B-ALL cells in the thymus of ΔPB mice. (**A**) Enlarged thymuses in ΔPB mice at various ages. The *y*-axis indicates percentage of mice with enlarged thymuses in the age groups indicated on the *x*-axis. (**B**) Increased thymic weight in ΔPB mice. The total weight of the thymus in grams was compared in ΔB (*n* = 7) and ΔPB mice (*n* = 9) between 19 and 23 wk of age. ***$p < 0.001$ by Student *t* test. (**C**) Expression of IL-7Rα by tumor cells in enlarged thymuses. The *y*-axis indicates percentage of mice in which the frequency of IL-7Rα–expressing thymocytes was >60%, in age groups indicated on the *x*-axis. (**D**) Coexpression of IL-7Rα with immature B cell markers in ΔPB mice. Flow cytometric analysis was performed to determine expression of the indicated markers. Increased brightness of IL-7Rα in the *third panel* is a consequence of using Cy7 rather than PE as a fluorophore. (**E** and **F**) Increased levels of total and phosphorylated Akt (pAkt) in ΔPB B-ALL cells. Immunoblotting for pAkt, total Akt (Akt), or β-actin was performed on lysates prepared from the spleen or thymus of a 16-wk-old ΔPB mouse, as well as from a ΔB control. Bone marrow cells cultured in IL-7 were used as a positive control for Akt activation. Result shown is representative of two experiments performed. (**G**) Progressive loss of B220 expression on leukemic B cells in enlarged thymus of ΔPB mice. The *y*-axis indicates the percentage of mice in each age group in which the enlarged thymus contained either B220^{+} cells (B220[pos]), B220^{−} cells (B220[neg]), or a mixture of both B220[+] and B220^{−} cells (mixed).
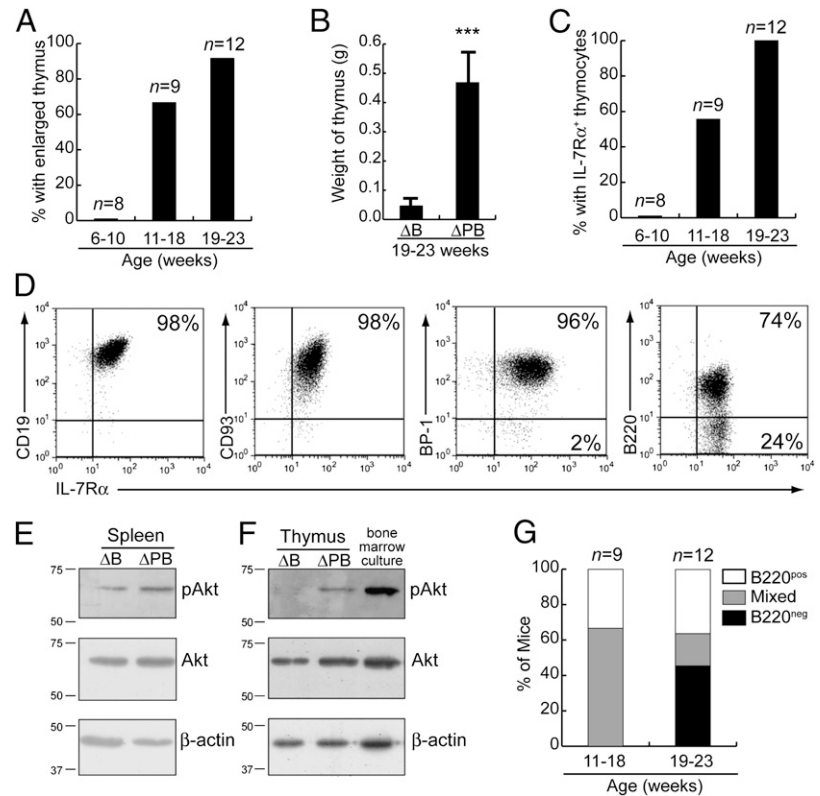
bar) was still extensively infiltrated with CD19^{+} IL-7Rα^{+} cells. CD19^{+} B-ALL cells were negative for c-Kit and expressed low levels of cell-surface IgM (data not shown). IL-7Rα^{+} B-ALL cells expressed the pre-B cell markers BP-1 (33), AA4.1/CD93 (34), and variable levels of B220 (Fig. 1D). To determine whether there was functional IL-7R signaling in IL-7Rα[+] B-ALL cells, we determined the status of Akt phosphorylation (35). Immunoblotting of lysates prepared from the tissues of 11- to 18-wk-old ΔPB mice showed increased levels of phosphorylated and total Akt protein compared with ΔB mice in either spleen (Fig. 1E) or thymus (Fig. 1F), suggesting active IL-7R signaling in these cells. B-ALL cells from 11- to 18-wk-old ΔPB mice were B220[+] or were a mixture of B220[+] and B220^{−} cells (Fig. 1D, *right panel*; Fig. 1G). However, >40% of B-ALLs from mice between 19 and 23 wk of age were exclusively B220[−] (Fig. 1G). These results suggest that expression of B220 on B-ALL cells was progressively lost as ΔPB mice aged. *Cd45*, encoding B220, has been shown to be a PU.1 target gene, so it is expected that the loss of B220 is a consequence of *Sfpi1* deletion (36). In summary, the incidence of thymus enlargement in ΔPB mice increased with age, and the B-ALL cells that infiltrated the thymus in ΔPB mice expressed functional IL-7R.

*IL-7–dependent pre-B cell lines can be established from the thymus of ΔPB mice*

Expression of functional IL-7R by B-ALL cells in ΔPB thymus suggested that these cells might proliferate in response to IL-7 in culture. We tested whether B-ALL cells from the enlarged thymus of 19- to 23-wk-old ΔPB mice could proliferate in response to complete media, ST2 stromal cells, IL-7, or ST2 stromal cells and IL-7. As controls, thymocytes from age-matched littermate ΔB mice were cultured under the same conditions. Control ΔB thymocytes did not grow in culture in any condition. In contrast, ΔPB cells proliferated in response to IL-7 alone or ST2 + IL-7

(Fig. 2A). Cells from the enlarged thymuses of 19- to 23-wk-old ΔPB mice generated colonies in IL-7–containing methylcellulose at a frequency of ∼50 per 50,000 cells, suggesting that IL-7 was sufficient to induce proliferation in a manner that did not require cell–cell contact. After expansion, these cells had the typical small and highly refractile appearance of pro-B/pre-B cells, and could be efficiently expanded and propagated as cell lines (Fig. 2B). ΔPB B-ALL cells proliferated in response to either IL-7–conditioned media or purified rIL-7 (Fig. 2C). However, established ΔPB B-ALL cell lines did not proliferate in response to cell culture media alone, stem cell factor, Flt3 ligand, IL-3, M-CSF, or GM-CSF (Fig. 2C). Cultured ΔPB B-ALL cells lines expressed CD19, BP-1, and AA4.1/CD93 (data not shown), as well as IL-7Rα and surface IgM (Fig. 2D). Cultured cells did not express detectable c-Kit but did express cell-surface pre-BCR as detected by Ab SL156 (Fig. 2D). Because c-Kit is considered a marker for pro-B cells that is lost on differentiation into pre-B cells (37), these results are most consistent with a description of IL-7–dependent cell lines established from ΔPB B-ALL as pre-B cells. In summary, IL-7 is both necessary and sufficient for the ex vivo growth of pre-B–like cell lines from the thymus of 19- to 23-wk-old ΔPB mice.

The Blnk gene is activated by PU.1 and/or Spi-B

We previously reported that B-ALL cells from ΔPB mice express reduced levels of mRNA transcripts encoding BLNK relative to control B cells (15). Because mutation of *Blnk* is sufficient to induce B-ALL in mice (23, 24), we sought to determine whether *Blnk* is a target of gene activation by PU.1, and Spi-B. IL-7–dependent ΔPB B-ALL cell lines expressed reduced levels of BLNK protein compared with wild type IL-7–dependent pro-B cells (Fig. 3A). ΔPB B-ALL cell lines expressed 10-fold reduced steady-state levels of *Blnk* mRNA transcripts compared with wild-type IL-7–dependent pro-B cells (Fig. 3B). Phylogenetic comparisons of

FIGURE 2. Establishment of IL-7–dependent cell lines from thymocytes of ΔPB mice. (**A**) IL-7 is sufficient to promote proliferation of ΔPB B-ALL cells. B-ALL cells from the enlarged thymus of 17-wk-old ΔPB or ΔB mice were placed in culture with IL-7 or IL-7 and ST2 stromal cells, and counted after 7 d. One representative experiment of five performed is shown. (**B**) Appearance of cultured ΔPB cells. Cells were photographed at ×40 original magnification using a Zeiss AxioVert inverted microscope under phase-contrast illumination. (**C**) IL-7 is both necessary and sufficient for proliferation of ΔPB B-ALL cells. Cells that had been grown for several passages in IL-7 were washed and cultured at 10^6 cells/flask in media containing cytokines indicated on the x-axis and counted after 4 d. (**D**) Cell-surface phenotype of IL-7–dependent ΔPB B-ALL cells. Flow cytometry was used to determine expression of the markers indicated on the x-axis. The open histogram indicates staining with isotype control Ab, whereas the filled histogram indicates staining with the indicated Ab.
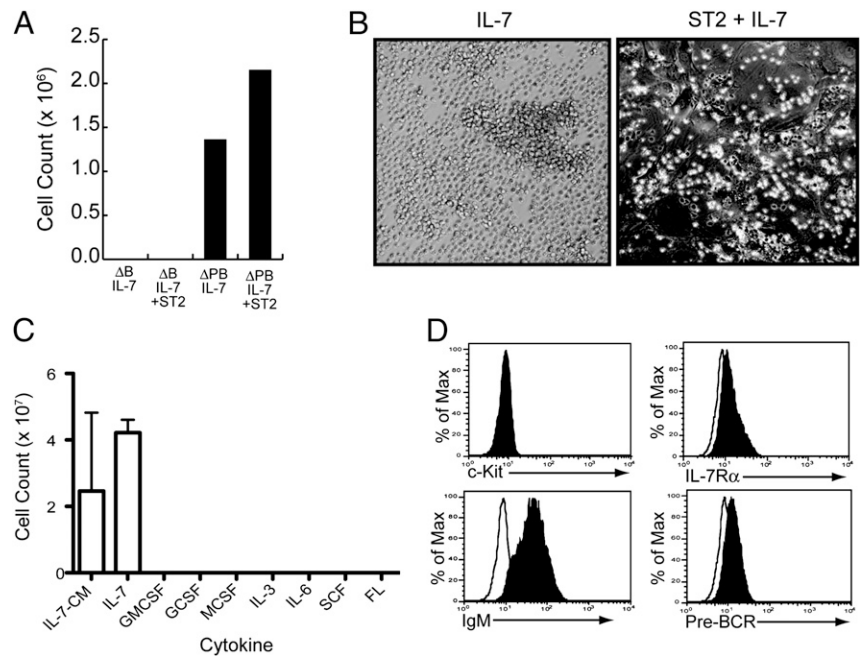
DNA sequences in the previously described mouse *Blnk* promoter region (38), as well as analysis using a position weight matrix algorithm (27), were used to identify predicted PU.1/Spi-B binding sites. Three PU.1 and/or Spi-B binding sites were predicted based on high matrix similarity scores (Fig. 4A). Interestingly, the site with the highest matrix similarity score was located near the published transcription start sites of the *Blnk* gene and near binding sites for Pax5 (38) (Fig. 4A). A 418-bp segment of the murine *Blnk* promoter that displayed high mouse–human similarity was amplified and cloned from C57BL/6 B cell genomic DNA. The *Blnk* promoter was tested for activity by transient transfection in WEHI-279 B lymphoma cells and showed activity only in the forward orientation (Fig. 4B and data not shown). Mutation of the highest scoring predicted binding site from GGAA to GGAC (Fig. 4C) reduced *Blnk* promoter activity by 3-fold (Fig. 4B). Therefore, the *Blnk* promoter contains a potential PU.1 and/or Spi-B binding site.

To determine whether PU.1 or Spi-B can directly interact with the *Blnk* promoter in B cells, we performed ChIP experiments.
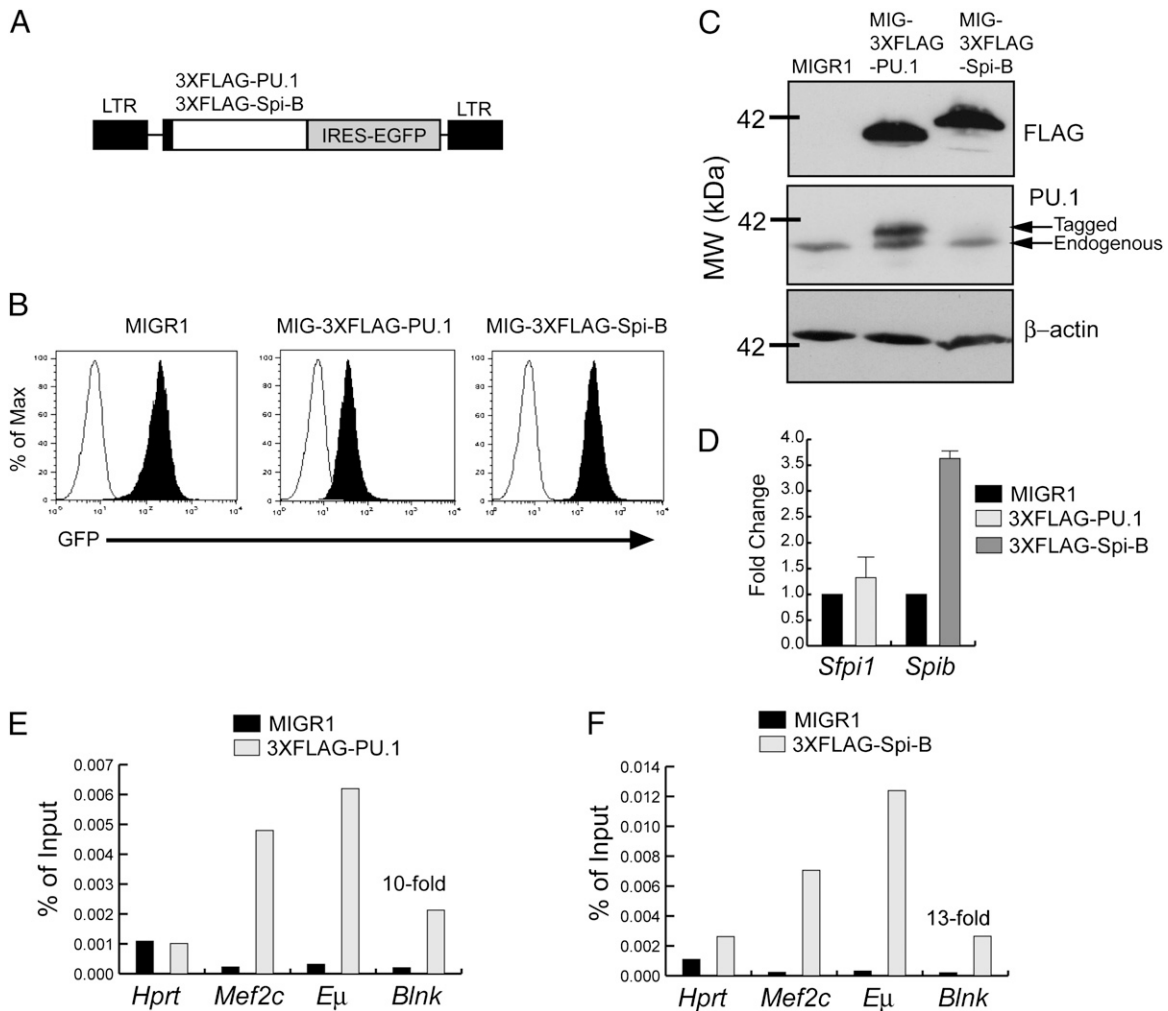
Because anti–Spi-B Abs were not available, WEHI-279 B lymphoma clones were generated expressing GFP and 3XFLAG-tagged full-length PU.1 or Spi-B protein under the control of a retroviral long terminal repeat promoter (Fig. 5A, 5B). As a negative control, a WEHI-279 clone expressing only GFP (MIGR1)
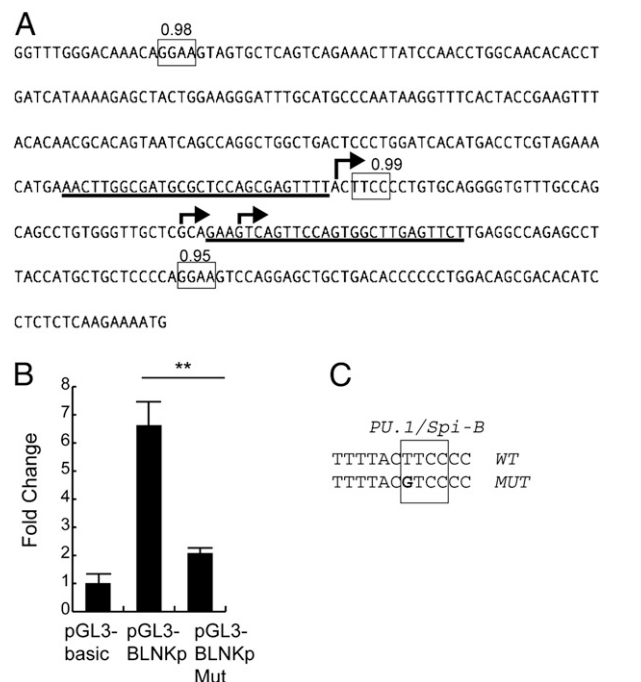
FIGURE 4. *Blnk* is transcriptionally activated by PU.1 and/or Spi-B. (**A**) Schematic of the *Blnk* promoter region. Major transcription start sites are indicated by arrowheads. Predicted PU.1/Spi-B binding sites are indicated by boxes. Numbers indicate matrix similarity scores. Underlined sequences represent previously identified Pax5 binding sites. (**B**) Reduced *Blnk* promoter activity after mutagenesis of a predicted PU.1/Spi-B binding site. The *Blnk* promoter fragment shown in (A) was tested for activity in a luciferase assay with either an intact PU.1/Spi-B binding site (BLNKp) or the site mutated as shown in (**C**) (BLNKp-Mut). **$p < 0.01$ by Student t test. (**C**) Site-directed mutagenesis of a predicted PU.1/Spi-B binding site. MUT, Mutated DNA sequence.

FIGURE 3. ΔPB B-ALL cells express reduced levels of BLNK. (**A**) Reduced levels of BLNK protein in ΔPB B-ALL cells. Immunoblot was used to compare relative levels of BLNK protein in two independent ΔPB B-ALL cell lines relative to IL-7–dependent wild type pro-B cells. As a loading control, levels of β-actin protein were determined by immunoblot (*lower panel*). (**B**) Reduced levels of *Blnk* mRNA transcripts in ΔPB B-ALL cells. RT-qPCR was used to compare relative levels of *Blnk* mRNA transcripts in ΔPB B-ALL cells compared with IL-7–dependent wild-type pro-B cells. Transcript levels were normalized to β2-microglobulin mRNA transcript levels. ***$p < 0.001$ by Student t test.

FIGURE 5. Interaction of PU.1 and Spi-B with the *Blnk* promoter. (**A**) Schematic of retroviral vectors. (**B**) Flow cytometric analysis of WEHI-279 clones expressing GFP only (MIGR1), FLAG-tagged PU.1 and GFP, or Spi-B and GFP. (**C**) Expression of FLAG-tagged proteins by WEHI-279 clones. Immunoblot was performed on lysates from the clones described in (A) using anti-FLAG Ab (*upper panel*), anti-PU.1 Ab (*center panel*), or anti–β-actin Ab (*lower panel*). (**D**) Increase in steady-state levels of transcripts encoding PU.1 and Spi-B in infected WEHI-279 cells. RT-qPCR was performed to determine relative levels of the indicated transcripts in WEHI-279 cells infected with control retrovirus (MIGR1) or retrovirus encoding PU.1 (3XFLAG-PU.1) or Spi-B (3XFLAG–Spi-B). (**E** and **F**) ChIP analysis. Chromatin prepared from the clones described in (A)–(D) was immunoprecipitated with anti-FLAG Ab. Immunoprecipitated DNA was quantified by relative qPCR using primers recognizing the hypoxanthine-guanine phosphoribosyltransferase promoter (*Hprt*), *Mef2c* enhancer (*Mef2c*), *IgH* intronic enhancer (*Eμ*), or *Blnk* promoter (*Blnk*). Amounts of immunoprecipitated DNA are expressed as percentage of input. Enrichment was calculated as the fold change in percentage of input. Results shown are representative of three independent experiments.

was also generated (Fig. 5B). WEHI-279 cells infected with MIG-3XFLAG-PU.1 retrovirus expressed 2- to 3-fold higher PU.1 protein levels and 1.3-fold higher *Sfpi1* mRNA transcript levels than controls (Fig. 5C, 5D). A WEHI-279 clone infected with MIG–3XFLAG–Spi-B was selected for expression of FLAG-tagged Spi-B at levels comparable with FLAG-tagged PU.1, and was found to express *Spib* mRNA transcripts at 3.6-fold higher levels than endogenous *Spib* transcript levels in control cells (Fig. 5C, 5D). ChIP was performed on fixed WEHI-279 clonal cell lines using anti-FLAG mAb. qPCR was performed on immunoprecipitated DNA to determine association of regulatory regions with 3XFLAG-tagged PU.1 or Spi-B. Enrichment was compared with DNA immunoprecipitated from MIGR1 cells using anti-FLAG Ab. The promoter of the *Hprt* gene, which was not expected to be associated with PU.1 or Spi-B, was not significantly enriched by immunoprecipitation with anti-FLAG Ab (Fig. 5D, 5E). In contrast, intronic enhancers of the *Mef2c* and *Igh* loci, which are highly expressed in the B cell lineage under control of PU.1 (39, 40), were enriched in cells expressing FLAG-tagged

PU.1 and Spi-B (Fig. 5D, 5E). PU.1 and Spi-B were associated with the *Blnk* promoter, showing enrichment by 10- and 13-fold compared with the negative control, respectively (Fig. 5D, 5E). Reanalysis of published ChIP-sequencing data using anti-PU.1 in murine B cells (41) confirmed that endogenous PU.1 interacts with the site we identified in the *Blnk* promoter (data not shown). Taken together, these results suggest that PU.1 and Spi-B activate *Blnk* transcription by direct interaction with the *Blnk* promoter.

Forced expression of BLNK opposes the growth of cultured ΔPB B-ALL cells

If transformation of ΔPB cells is, in part, a consequence of reduced BLNK expression, then restoration of BLNK expression might be expected to oppose growth of these cells in vitro. To test this idea, we used RT-PCR to amplify the full *Blnk* coding sequence from C57BL/6 splenic B cell RNA. *Blnk* cDNA was cloned and ligated into the retroviral vector MIGR1 to promote expression of BLNK protein, as well as enhanced GFP under control of the retroviral long terminal repeat promoter (Fig. 6A).
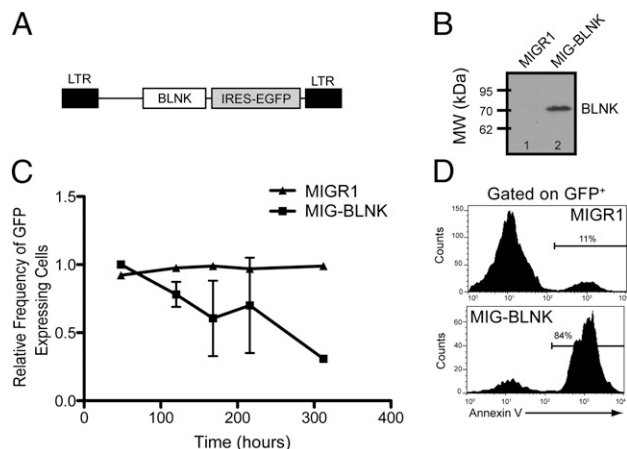
FIGURE 6. Forced expression of BLNK opposes IL-7–dependent proliferation of ΔPB B-ALL cells. (**A**) Schematic of the MIG-BLNK retroviral vector. (**B**) Expression of BLNK protein in NIH-3T3 cells infected with retroviral vectors. Immunoblot was used to determine BLNK expression in NIH-3T3 cells infected with either MIGR1 or MIG-BLNK. (**C**) Reduced frequency of MIG-BLNK–infected ΔPB B-ALL cells upon serial passage. Flow cytometry was used to determine the percentage of cells infected with MIGR1 (triangles) or MIG-BLNK (squares) at the time points shown on the *x*-axis. The *y*-axis shows relative infection frequencies normalized to the frequency of infected cells at 48 h. (**D**) Increased apoptosis of MIG-BLNK–infected ΔPB B-ALL cells 48 h postinfection. Cells were stained with Annexin V and analyzed using flow cytometry. Results are shown gated on GFP⁺ cells.

BLNK protein was expressed in NIH-3T3 cells infected with the MIG-BLNK retrovirus (Fig. 6B). To determine whether forced BLNK expression opposes proliferation of cultured ΔPB B-ALL cells, we infected cultured ΔPB cells with MIG-BLNK retrovirus or with MIGR1 retrovirus as a control. Infected cells were cultured 48 h to promote retroviral integration, and the frequency of cells expressing GFP was determined by flow cytometry on passage every 72–96 h. The relative frequency of MIGR1-infected cells changed no more than 1.2-fold for the duration of the experiment. In contrast, the relative frequency of MIG-BLNK–infected cells was reduced with each passage until termination of the experiment (Fig. 6C). This result suggested that BLNK-infected ΔPB cells proliferated poorly compared with MIGR1-infected cells. To confirm this finding, we placed MIGR1- or MIG-BLNK–infected ΔPB cells in methylcellulose colony-forming assays with IL-7. After 7 d, GFP⁻ and GFP⁺ colonies were counted using an inverted fluorescence microscope. The frequency of GFP⁺ colonies generated by MIGR1-infected cells was similar to the frequency of GFP⁺ cells placed in the assay as determined by flow cytometry. In contrast, no GFP⁺ colonies were generated from MIG-BLNK–infected ΔPB cells. To determine why BLNK-infected ΔPB cells failed to proliferate, we measured early apoptosis using Annexin V staining 48 h after retroviral infection. A high frequency of MIG-BLNK–infected cells stained positively with Annexin V compared with MIGR1-infected cells (Fig. 6D). We conclude that restoration of BLNK expression using a retroviral vector opposes IL-7–dependent proliferation of ΔPB B-ALL cell lines, at least, in part, by inducing early apoptosis.

Discussion

This study explored the mechanism by which deletion of genes encoding PU.1 and Spi-B induces B-ALL in mice. We showed that B-ALL cells from the enlarged thymus of ΔPB mice express IL-7R on their surface and can be grown ex vivo in response to IL-7. Second, we showed that the gene encoding BLNK is a direct target

of activation by PU.1 and/or Spi-B. These results suggest that PU.1 and Spi-B are complementary activators of *Blnk* transcription, and that B-ALL is induced in ΔPB mice, at least in part, as a consequence of reduced BLNK levels.

PU.1 and Spi-B have both been implicated as oncogenes or tumor suppressors, depending on the circumstance. PU.1 was discovered as a gene upregulated in murine erythroleukemia as a consequence of proviral insertion by the murine SFFV retrovirus (42). Overexpression of PU.1 has also been implicated as a cause of T cell leukemia in genetically modified mice (43). In contrast, reduced levels of PU.1 caused by mutation or repression of the *Sfpi1* gene are sufficient to induce acute myeloid leukemia (8, 44). Spi-B is frequently overexpressed in diffuse large B cell lymphoma (45). Therefore, PU.1 and Spi-B have known functions as either oncogenes or tumor suppressors, but little is known about the mechanism of these functions. We expect that PU.1 and Spi-B transcriptionally activate downstream target genes with tumor-suppressor function.

BLNK is considered a tumor suppressor because: 1) point mutations or reduced levels of BLNK are associated with human B-ALL (21–23), and 2) mutation of *Blnk* is sufficient to induce B-ALL in mice (24, 46). However, BLNK is required for B cell development in mice and humans, causing primary human B cell deficiency when mutated (16, 20). BLNK is required for B cell development because it links signals from the pre-BCR or BCR with B cell differentiation by functioning as an adaptor or scaffold to promote interaction of key signaling proteins (20). Several recent studies suggest that BLNK has a dual function of enforcing BCR signaling whereas blocking IL-7 signaling (35, 46, 47). BLNK interacts directly with JAK3, promoting the uncoupling of JAK3 from IL-7 signaling (46). BLNK also inhibits activation of the PI3K-Akt pathway downstream of IL-7R signaling (35, 47). Therefore, reduced BLNK levels, as might be induced by mutation, repression, or reduced transcriptional activation, would be expected to result in increased IL-7R signaling, as well as reduced BCR signaling, promoting proliferation and impairing differentiation (35, 46).

Addiction to IL-7 signaling may be a common feature of pediatric leukemia. Human B-ALL tumor cells frequently express functional IL-7R on their surface and can respond to IL-7 in culture (48). Gain-of-function mutations in the *IL7R* gene (encoding IL-7Rα) are associated with pediatric ALL (49). In mice, overexpression of IL-7 is sufficient to induce lymphoproliferation leading to leukemia (50). As described in *Results*, B-ALL cells in ΔPB mice express high levels of IL-7R on their surface and grow readily in response to IL-7 ex vivo. These results suggest that IL-7R expression and IL-7 signaling plays a role in development of leukemia in ΔPB mice. Developing B cells express high levels of IL-7Rα, but this receptor is not expressed by mature B cells (51). Little is known about what factors control transcriptional downregulation of *IL7R* in developing B cells; however, we speculate that BLNK might be involved in regulation of IL-7Rα expression, as well as downstream signaling. B-ALL cells from *Blnk*⁻/⁻ mice express IL-7R on their surface and can be efficiently propagated in culture in response to IL-7 (23, 24). In addition, *Blnk*⁻/⁻ B-ALL cells grow in autocrine fashion, both producing and proliferating in response to IL-7 (46). Therefore, in the absence of BLNK, pre-B cells might have sustained IL-7R signaling as a consequence of a failure to inhibit IL-7R signaling, as well as a failure to appropriately downregulate IL-7Rα expression.

Our findings suggest that activation of BLNK expression is an important pathway by which PU.1 and/or Spi-B regulate B cell differentiation. B cells deficient in PU.1 and/or Spi-B have severely impaired BCR signaling (52) and, as a consequence, cannot

efficiently differentiate into follicular B cells (31). PU.1 and Spi-B regulate a number of genes required for BCR signaling, including P2Y10, Grap2, and Btk (53, 54). Interestingly, mutation of *Btk* strongly cooperates with mutations in *Blnk* to induce B-ALL in mice (55). These results suggest that PU.1 and Spi-B increase BLNK and Btk levels to enforce B cell differentiation and oppose oncogenic transformation.

B-ALL is frequently associated with chromosomal translocations that result in generation of abnormal transcription factors. Twenty-five percent of cases of pre-B ALL involve the t(12;21) chromosomal translocation that results in an *ETV6-RUNX1* (also known as *TEL-AML1*) fusion gene (1). The significance of this observation to this study is that RUNX1 is an important upstream activator of the *SPI1* gene in humans and the *Sfpi1* gene in mice (56). PU.1 levels are reduced upon expression of RUNX1 fusion proteins such as ETO-RUNX1 (57, 58). Recently, it was also shown that B-ALL cells harboring *ETV6-RUNX1* fusions express reduced levels of Spi-B (14). Based on these observations, we wish to propose a hypothetical model for B-ALL leukemogenesis involving PU.1 and Spi-B. First, chromosomal translocations resulting in the generation of *ETV6-RUNX1* fusion protein in 25% of all B-ALL patients may result in reduced PU.1 and Spi-B levels as a consequence of reduced *SPI1* and *SPIB* transcription. Reduced levels might additionally cooperate with loss-of-function *SPI1* mutations that have been observed (7). Reduced levels of PU.1 and Spi-B in pre-B cells would result in changes in gene expression, including reduced *BLNK*, leading to increased IL-7–dependent proliferation. Increased proliferation and/or expansion of pre-B cells may lead to B-ALL by additional mechanisms including genomic instability. Interestingly, BLNK has also been implicated in the maintenance of genomic integrity (59). Further consideration of this pathway of leukemic transformation may result in identification of checkpoints that could be evaluated for molecular targeted therapy.

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