

Fatal Acute Lymphoblastic Leukemia in Mice Transgenic for B Cell-Restricted *bcl-x_L* and *c-myc*¹

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Expression of the *c-myc* gene is frequently dysregulated in malignant tumors and translocations of *c-myc* into the Ig H chain locus are associated with Burkitt's-type lymphoma. There is indirect evidence that *bcl-x*, an anti-apoptotic member of the *bcl-2* gene family, may also contribute to a variety of B lymphoid tumors. In this study, we show that mice transgenic for both B cell-restricted *c-myc* and *bcl-x_L* developed aggressive, acute leukemias expressing early B lineage and stem cell surface markers. Of interest, the tumor cells proliferated and differentiated down the B cell developmental pathway following in vitro treatment with IL-7. Analysis of sorted leukemic cells from spleen indicated constitutive expression of sterile μ and κ transcripts in combination with evidence for D-J_H DNA rearrangements. Several B cell-specific genes were either not expressed or were expressed at low levels in primary tumor cells and were induced following culture with IL-7. IL-7 also increased V-J κ and V-DJ_H rearrangements. These data demonstrate oncogenic synergy between *c-myc* and *bcl-x_L* in a new mouse model for acute lymphoblastic leukemia. Tumors in these animals target an early stage in B cell development characterized by the expression of both B lineage and stem cell genes. *The Journal of Immunology*, 2004, 172: 6684–6691.

In 1981, Hayward et al. (1) showed that avian leukemia virus-associated lymphomas were caused by retroviral insertion into the 5' regulatory region of the *c-myc* gene. Shortly thereafter, Dalla-Favera et al. (2) demonstrated that *c-myc* was involved in the chromosomal t(8;14) translocations found in nearly all human sporadic and endemic Burkitt-type lymphomas (2), while Shen-Ong et al. (3) reported the involvement of *myc* translocations in mouse plasmacytomas (3). Since that time, there has been significant interest in understanding the mechanism by which *Myc* leads to cellular transformation (4).

Myc is a transcription factor of the B-zip helix-loop-helix family (5). *Myc* binds DNA E box elements containing the core sequence CAC(G/A)TG as an obligate heterodimer with Max, or other factors, and can function as either a transcriptional activator or repressor. The *myc* gene itself is transcriptionally up-regulated during normal proliferation and repressed in cells undergoing differentiation (6). Target genes for *myc* include ornithine decarboxylase (7) and the cell cycle phosphatase Cdc25A (8), either of which when overexpressed in cell culture at least partially mimic the transforming activity of *Myc*. Dysregulated expression of *Myc* causes cell cycle progression and proliferation, and, in addition, also induces apoptotic cell death (9). It has been proposed that these two functions of *Myc*, proliferation and apoptosis, are inher-

ently linked processes (10). Dysregulated *Myc* expression along with blockade of *Myc*-induced death signaling, for instance by coexpression of the antiapoptotic gene *bcl-2* (11, 12), is sufficient to induce malignant transformation of cells in a variety of systems.

Mice transgenic (Tg)³ for *Myc* within the B lineage (*E μ -myc*) have provided a useful model system to investigate the role of this oncogene in lymphomagenesis. Animals carrying the *E μ -myc* transgene harbor a large population of rapidly proliferating, but nontransformed, polyclonal pre-B and surface IgM⁺ B cells for the first several months of life (13, 14). This is followed by the subsequent development of highly malignant clonal B cell lymphomas that are invariably lethal, with mice dying between 4 and 7 mo of age. The clonal nature of the fatal lymphomas in these mice in combination with the variable timing of tumor onset is consistent with spontaneous secondary genetic mutations, and such mutations have been documented in *ras*, *ARF*, and *p53* (15, 16). This model has also been used to show synergy between *bcl-2* and *c-myc* in malignant transformation of B lineage cells (17, 18). Mice Tg for both a B cell-restricted *bcl-2* transgene along with the *E μ -myc* transgene developed highly malignant lymphomas in the first few weeks of life. These tumors expressed markers of early hemopoietic cells and could be serially transplanted in recipient mice (17). Cell lines derived from these tumors were able to differentiate down either the B lymphoid or macrophage developmental pathways following appropriate stimulation with cytokines and/or LPS (18).

Bcl-x is a member of the *bcl-2* family of death regulatory genes (19), and *bcl-x_L*, the long isoform of the gene, has potent death inhibitory activity. *Bcl-x_L*, like *bcl-2*, is thought to inhibit cell death by protecting the mitochondrial membrane from release of cytochrome *c* following various apoptotic stimuli (20). *Bcl-x_L* prolongs the life span of transfected cells and renders such cells resistant to death following incubation with many inducers of apoptosis in vitro (19, 21). B and T cells from lymphocyte-targeted

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³ Abbreviations used in this paper: Tg, transgenic; ALL, acute lymphoblastic leukemia; Rag, recombination-activating gene; SPF, specific pathogen free; PAS, periodic acid-Schiff; MN, Minnesota.

bcl-x_L Tg mice also show resistance to cell death *in vivo* (22–24). For instance, Tg overexpression of *bcl-x_L* in B cells leads to the accumulation of large numbers of pro-B cells with failed V(D)J rearrangements (22) and allows self-reactive B cells to survive in the presence of membrane-bound self-Ag (25).

Bcl-x_L is overexpressed in several solid tumors (26–28) as well as hemopoietic tumors including acute myelogenous leukemia (29), Hodgkin's lymphoma (30), non-Hodgkin's lymphoma (31), HIV-associated lymphoma (32), and myeloma (33). Recently, translocations and rearrangements of *bcl-x* have been demonstrated in murine B and T cell tumor lines (34).

In the studies described here, we bred *Eμ-c-myc* Tg mice with animals overexpressing B cell-restricted *Bcl-x_L*. *Bcl-x_L/myc* double Tg animals developed highly malignant and fatal leukemia, with the transformed cells representing a very early stage in mouse B cell development. These mice provide a new model for the study of acute lymphocytic leukemia (ALL), and demonstrate oncogenic synergy between *bcl-x_L* and *c-myc*.

Materials and Methods

Mice

Heterozygote *bcl-x_L* B cell Tg mice (22) and *Eμ-c-myc* Tg mice (13), both fully backcrossed onto the C57BL/6 genetic background, were bred in the specific pathogen-free (SPF) animal facility at the University of Minnesota (Minneapolis, MN). Genotyping for the *bcl-x* transgene was performed as described previously (22). The *c-myc* transgene was detected by PCR using the forward primer 5'-CGCAAGGATAGGTCGAATTT and the reverse primer 5'-GCCCGTTAAAGCTACCAGTT. Double Tg mice were sacrificed when they developed signs and symptoms of advanced malignancy (ruffled fur, growth arrest, tachypnea, hunched posture) and whenever possible were used immediately for experiments. All animals were housed in a SPF facility at the University of Minnesota, and all experiments were approved by the University of Minnesota Animal Care Committee.

Cell preparation

Bone marrow (BM), spleen, and blood were obtained as described elsewhere (25, 35). Briefly, single-cell suspensions of BM (from both femurs and tibias) were prepared by flushing the marrow cavities with wash buffer (1× RPMI 1640 with glutamate, 2.5% FCS). Spleen preps were obtained by gentle dissociation of whole spleens followed by filtering through a cell strainer. Peripheral blood was obtained by venous puncture or at autopsy, and RBC were depleted by ACK lysis.

Staining, flow cytometry, and cell sorting

The following directly conjugated mAbs were used for cell staining: B220-Cy, CD43-PE, Sca-1-FITC, CD4-Cy, Gr-1-FITC, Thy 1.2-PE, CD19-PE, IgM-PE, IgD-FITC, CD34-FITC, Mac-1-FITC, CD3-PE (all Abs purchased from BD Pharmingen, San Diego, CA). Standard flow cytometric analysis was conducted on a FACSCalibur machine (BD Biosciences, Mountain View, CA) at the University of Minnesota Cancer Center, and data were analyzed using CellQuest (BD Biosciences) and FlowJo (TreeStar, Ashland, OR) software. For sterile sorting, splenic cells were stained with anti-B220, anti-Sca-1, and anti-CD43 mAbs, and samples were held at 4°C during the sterile sort. Reanalysis of sorted fractions showed purities in excess of 98%.

Serial transplantation

Splenic cells (10⁵ in 100 μl of PBS) from a double Tg mouse (CD45.2 B6 allotype) were injected into the peritoneal cavity of CD45.1-congenic B6 recipient mice. Approximately 4–6 wk later the recipient animals showed signs of malignancy and were sacrificed for the next round of transplantation. The allotype difference allowed unequivocal identification of the transplanted tumor cells and was used for sorting of transplanted leukemic cells.

Cell culture

Splenic cells (~90% leukemic) from *c-myc/bcl-x_L* double Tg mice were cultured in 50% RPMI 1640/50% EHA medium containing 10% heat-inactivated FCS (HyClone, Logan, UT), 50 μM 2-ME, 2 mM L-glutamine, and antibiotics at 37°C in a humidified atmosphere with 5% CO₂. Cells were cultured at an initial concentration of 0.5 × 10⁶ cells/ml with 50 U/ml

rIL-7 (R&D Systems, Minneapolis, MN). Cultured cells were harvested between 12 and 16 days for flow cytometric analysis and preparation of mRNA and genomic DNA.

RT-PCR and PCR assays

All semiquantitative RT-PCR were validated and performed as previously described (22, 25, 36). Poly(A)⁺ mRNA was extracted from 3 to 5 × 10⁶ fresh sorted cells and from 2 to 5 × 10⁶ IL-7 cultured cells using a MicroFast Track kit (Invitrogen, San Diego, CA). mRNA was stored as an ethanol precipitate at –80°C until ready for use. Two hundred nanograms of each sample was used for randomly primed first-strand cDNA synthesis using a cDNA Cycle kit (Invitrogen). One-twentieth of each cDNA sample was used for each semiquantitative PCR using gene-specific primer pairs (*β-actin*, recombination-activating gene (*Rag*) 1, *Rag*2, *TdT*, *λ5*, *VpreB* (37); *CD19* (38); sterile *μ*, sterile *κ* (39); *B29*, *BSAP*, *Id*, and *E47* (40)). PCR was performed in 20-μl volumes containing 1 μl of cDNA, 1× PCR buffer, 2.5 mM MgCl₂, 200 mM dNTPs, 100 ng of each sense and antisense primer, and 1 U of AmpliTaq Gold (PerkinElmer, Wellesley, MA). After an initial 10-min incubation at 95°C, the PCR was conducted as follows: denaturation at 95°C for 30 s, annealing at 60°C for 30 s (for the first five cycles) and 55°C (for subsequent cycles), and extension at 72°C for 45 s. Cycles (ranging from 22 to 30) were optimized so that amplification was in the linear range. The annealing temp was 64°C for the *CD19* amplification and 60°C for sterile *μ* and sterile *κ*. Aliquots of the PCR samples were then electrophoresed through 1% agarose gels and blotted onto nitrocellulose membranes. Following UV cross-linking, the blots were hybridized overnight with either a randomly primed radiolabeled cDNA probe at 42°C (for *β-actin*, *Rag*1, *Rag*2) or a [³²P]ATP end-labeled oligonucleotide internal probe at 56°C. Hybridization probes used were as follows: *TdT*, AGC TGC AGA ACA TCA AAG CCA GCT C; *λ5*, AGG CTA GAA TGA GTG ACT GGG AAG G; *VpreB*, ACC CTG AGC AAC GAC CAT AAC ATT GG; *CD19*, TCC CTG GGC ATC TTG CTA GTG ATT G; sterile *μ*, AGC ACC ATT TCC TTC ACC TGG AAC T; sterile *κ*, TCC AGT GAG CAG TTA ACA TCT GGA G; *B29*, TAC CAG CAA TGA CAA GCA GTG ACC T; *BSAP*, GTC CGC CAA AGG ATA GTG GAA CTT G; *Id*, GGT ACT TGG TCT GTC GGA GCA AAG C; and *E47*, ACT CCT TTA GCG TAG TCA TCG GCT G. Following hybridization, membranes were washed and subjected to autoradiography. Genomic DNA was isolated from sorted tumor cells and *in vitro*-cultured cells using a high salt precipitation protocol. One hundred fifty nanograms of DNA (from ~40,000 cells) was used for each PCR. The *DH-JH2*, *V-DJH2*, *V-Jk1*, and control *Cκ* assays were performed as previously described (22, 25, 36).

Results

Bcl-x_L and *c-myc* synergize in leukemogenesis

Bcl-x_L B cell Tg mice (22) have been observed up to 2 years of age and do not exhibit an accelerated incidence of lymphoid malignancies compared with non-Tg littermates. Thus, similar to *bcl-2* B cell transgenics (41), the frequency of lymphomas in mice Tg for *bcl-x* alone appears to be low. To test the hypothesis that *bcl-x_L* could synergize with *c-myc* to induce malignant transformation of B lineage cells, we initiated a series of breedings between *bcl-x_L* and *Eμ-c-myc* Tg mice. All mice were on the C57BL/6 genetic background and were housed in SPF conditions.

Fig. 1 shows curves for the times to death or terminal disease for the non-Tg control littermates (B6), *Bcl-x_L* single Tg (*Bcl-x*), *c-myc* single Tg (*Myc*), and *bcl-x_L/c-myc* (*Bcl-x/Myc*) double Tg animals. There were no deaths of either B6 or *bcl-x_L* Tg animals during the observation period. The median survival for *c-myc* Tg animals was 20 wk, with animals first developing a polyclonal expansion of sIgM[–] pre-B cells and then succumbing to a clonal pre-B or mature B cell lymphoma, as previously described (13, 14). Strikingly, mice Tg both *bcl-x_L* and *c-myc* had a significantly shortened median survival of only 6 wk compared with *c-myc* mice, with all of the double Tg animals dying before 10 wk of age. The double Tg animals could usually be identified by the second or third week of life due to their small size, a hunched posture, and ruffled fur.

Examination of blood, BM, and spleens from 4- to 6-wk-old *bcl-x_L/c-myc* double Tg animals demonstrated the presence of an aggressive leukemia, with circulating leukemic cells in blood and

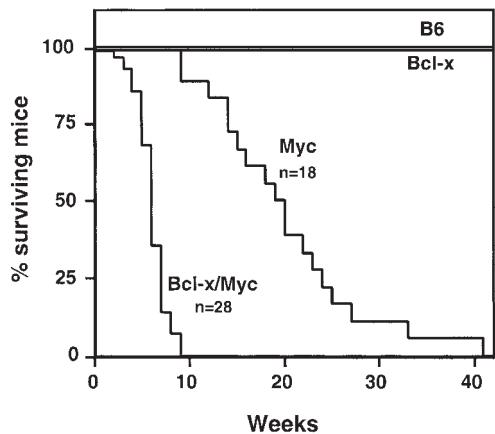


FIGURE 1. Accelerated death in *bcl-x_L/c-myc* double Tg mice. Shown are Kaplan-Meier survival curves for *c-myc* (Myc) single Tg, *Bcl-x_L* (*Bcl-x*) single Tg, *Bcl-x_L/c-Myc* (*Bcl-x/Myc*) double Tg, and control C57BL/6J (B6) mice. The median survival for *Bcl-x/Myc* double Tg mice was 6 wk, compared with 20 wk for Myc single Tg animals ($p < 1 \times 10^{-10}$ by paired *t* test).

extensive leukemic infiltration of lymphoid organs (BM, spleen, lymph nodes). As shown in Fig. 2, the blood of *bcl-x_L/c-myc* double Tg mice contained high numbers of lymphoid blasts (Fig. 2, A and B), many of which exhibited large cytoplasmic vacuoles (Fig.

2B). Of interest, similar vacuoles are often observed clinically in the leukemic cells of patients with Burkitt's lymphoma (42), although classically Burkitt's cells contain multiple small vacuoles. Staining of the leukemic cells from *bcl-x_L/c-myc* mice with periodic acid-Schiff (PAS) revealed a "block-like" pattern of cytoplasmic staining in nearly all tumor cells (Fig. 2C, small arrows), a finding characteristic of human ALL (43).

The double Tg spleens were markedly enlarged (cf Fig. 2, D and G), and the normal architecture of the spleen was severely disrupted with poorly defined follicles and extensive infiltration of the red pulp with tumor cells (Fig. 2, D–F). There was also evidence in most mice for extramedullary hemopoiesis in the double Tg spleens characterized by the presence of megakaryocyte progenitors and myeloid blasts. Leukemic infiltrates were present in most internal organs examined (e.g., thymus, lung, liver, and kidney; data not shown), generally most prominent in perivascular regions. Visible tumor nodules were often observed on the surface of the liver. Morphologically, the leukemic cells were large cells containing prominent nuclei with a loose chromatin configuration (Fig. 2B). Only a thin margin of cytoplasm could be visualized in the tumor cells by light microscopy. These morphologic features are typical of human ALL (44).

Leukemic Bcl-x_L/myc tumor cells are large and cycling

Flow cytometric examination of BM cells showed that the B220⁺ cells of both *myc* and *bcl-x_L/myc* Tg animals were larger, as measured

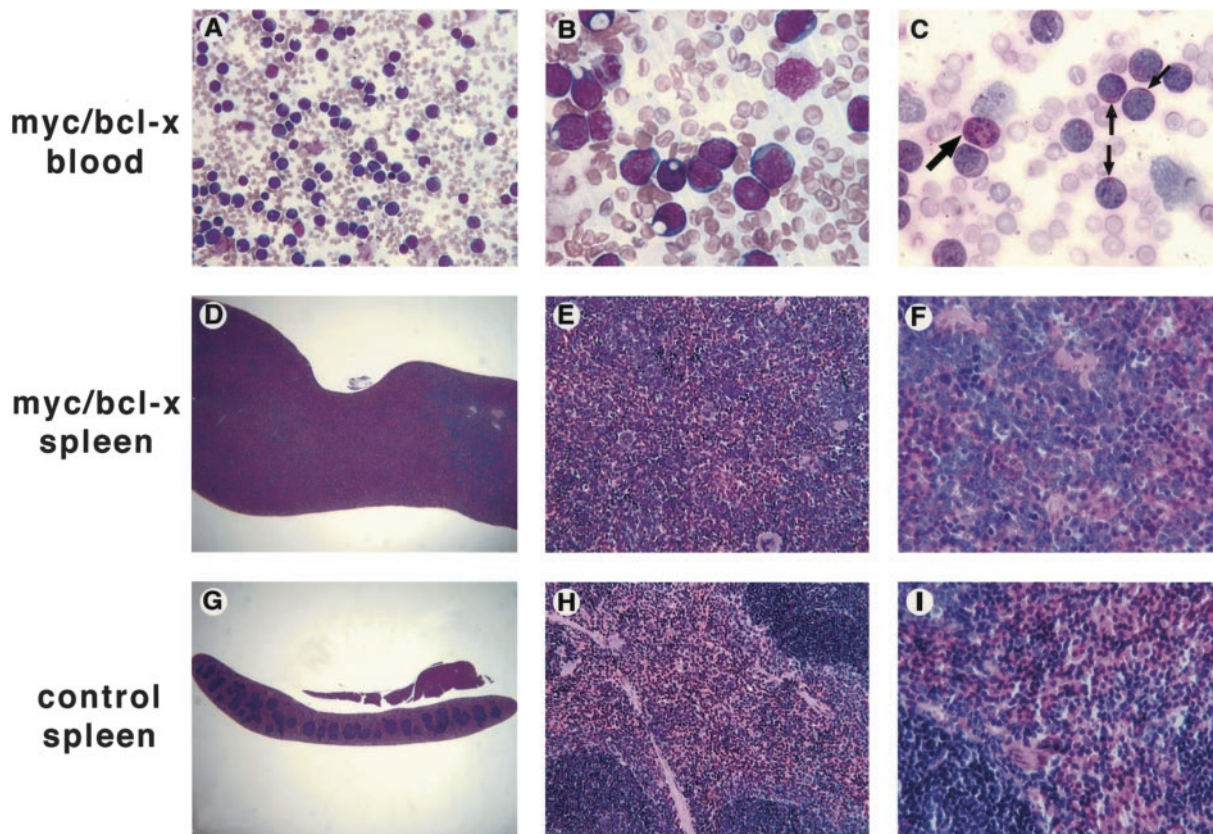


FIGURE 2. *Bcl-x_L/c-myc* double Tg mice develop fatal ALL. A and B, Wright's stain of peripheral blood from a *bcl-x_L/c-myc* double Tg animal with advanced disease. Essentially all of the nucleated cells in the blood were malignant blasts, and many of the leukemic cells contained large cytoplasmic vacuoles. Normal peripheral blood does not contain blasts. Peripheral blood white blood cell counts were at least 10-fold higher in *bcl-x_L/c-myc* animals than in normal mice. C, The leukemic cells show positive cytoplasmic staining for PAS (small arrows), a finding also described in human ALL. The cytoplasm of neutrophils (large arrow) serves as a positive control for the PAS stain. D, *Bcl-x_L/c-Myc* double Tg spleens are markedly enlarged compared with normal B6 spleens (G) and show a near complete disruption of the normal splenic architecture; compare tumor histology (E and F) with normal B6 spleen (H and I). Original magnifications: D and G, $\times 10$; E and H, $\times 200$; A, F, and I, $\times 400$; B and C, $\times 1000$.

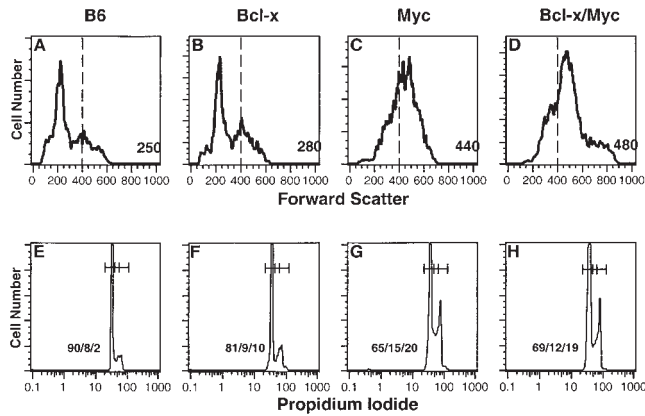


FIGURE 3. *Bcl-x_L/c-Myc* double Tg tumor cells are large and rapidly proliferating. *A-D*, Gated BM B220⁺ cells were analyzed by forward scatter in each of the indicated mice by flow cytometry. The dotted line serves as a reference point for each of the samples. The numbers shown in each graph refer to the mean forward scatter value. *E-H*, In parallel, permeabilized B220⁺ cells were stained with propidium iodide and the percentage of cells in the G₀-G₁/S/G₂-M phases of the cell cycle were determined. Representative of three independent experiments.

by forward light scatter, than those found in control or *bcl-x_L* Tg animals (Fig. 3). The median size of B220⁺ cells in *bcl-x_L/c-myc* Tg mice was slightly greater than *myc*-alone Tg cells. Examination of cell cycle status of BM B220⁺ cells by propidium iodide staining showed that although ~10% of control B220⁺ cells were in S or the G₂-M phases of the cell cycle, 20% of *bcl-x_L* Tg cells were in cycle, and 35 and 31% of *myc* and *bcl-x_L/c-myc* double Tg cells, respectively, were cycling (Fig. 3). These results indicate that the double Tg tumor cells are rapidly cycling *in vivo* at rates similar to *c-myc*-alone Tg B cells.

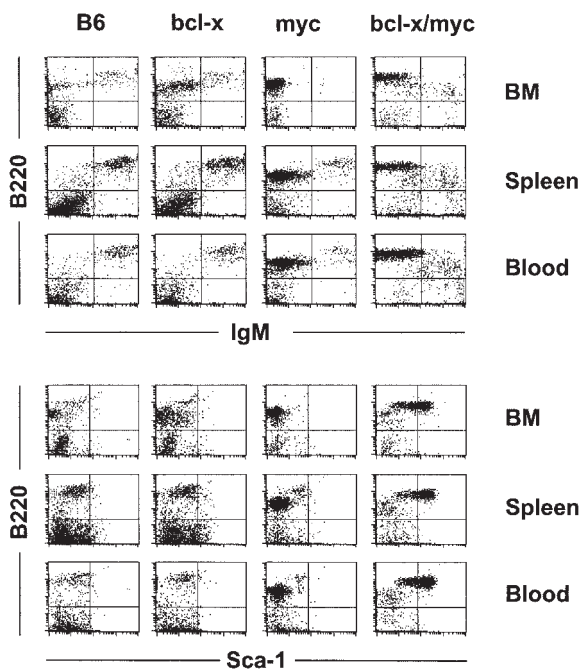


FIGURE 4. Tumor cells are IgM^{neg} Sca-1⁺. Single-cell preparations of BM, spleen, and blood from each of the indicated mice were analyzed by flow cytometry for surface expression of B220, IgM, and Sca-1. Results show lymphoid cells gated by forward and side scatter from a representative group of animals.

Cell surface phenotype of leukemic cells

We next used flow cytometry to determine the cell surface phenotype of the leukemic cells in *bcl-x_L/myc* double Tg animals. A representative experiment (of >20 performed) is shown in Fig. 4. The upper panel (Fig. 4) shows that the *myc/bcl-x* animals had a paucity of B220^{high}IgM^{high} B cells in BM, spleen, or blood, and instead exhibited a dominant population of IgM⁻ cells. Furthermore, most of the leukemic cells expressed B220 and high levels of the stem cell marker Sca-1, whereas Sca-1^{high} cells were very rare in any of the other mice.

Additional flow cytometric analysis revealed that the leukemic cells also expressed the B cell markers CD43 and AA4.1, the T cell markers CD4 and Thy1.2, and the myeloid marker Gr-1 (Fig. 5*B* and data not shown). The tumor cells did not express significant levels of CD19, IgM, IgD, CD3, Mac-1, CD34, *c-kit*R, or heat-stable Ag (Fig. 5 and data not shown). The tumors from >20 *bcl-x_L/c-myc* mice examined exhibited a virtually identical cell surface phenotype. Together, these data indicate that within the first few weeks of life *bcl-x_L/c-myc* double Tg mice develop a highly malignant and fatal leukemia, with the tumor cells expressing surface markers characteristic of several hemopoietic lineages.

Serial transplantation of leukemic cells

A series of experiments were next performed to determine whether the leukemic cells could be serially transplanted in syngeneic animals. The recipient mice used were congenic for the allotype marker CD45.1, so that the tumor cells (derived from CD45.2 allotype C57BL/6 mice) could be readily distinguished from host cells. Following *i.p.* injection of recipient mice with 10⁵ or 10⁶

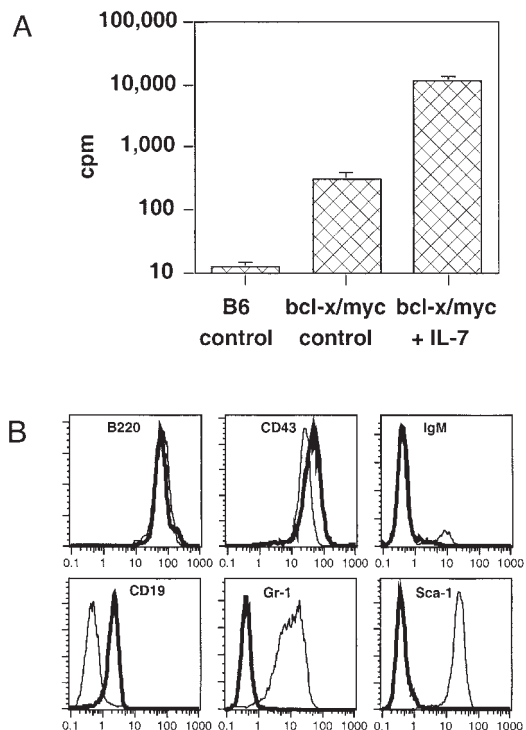


FIGURE 5. IL-7 responsiveness of tumor cells. *A*, Splenic B220⁺ cells from a B6 control mouse and a *Bcl-x_L/c-Myc* double Tg animal (*bcl-x/myc*) were incubated alone or with 50 U/ml rIL-7 for 3 days. [³H]Thymidine uptake was measured for the last 16 h of culture. Representative of five independent experiments. *B*, Histograms show the flow cytometric cell surface phenotype of tumor cells grown in IL-7 for 12 days and stained with the indicated labeled Abs. Fresh tumor cells, thin line; cultured cells, thick line. Representative of five experiments.

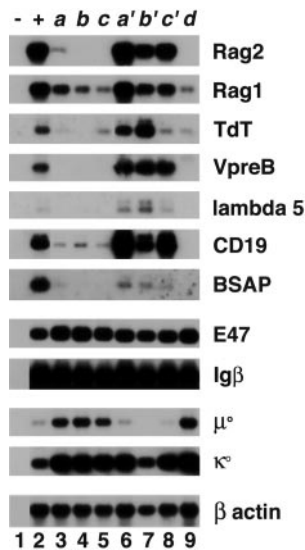


FIGURE 6. Induction of B cell-specific gene expression in tumor cells grown in IL-7. RT-PCR assays of gene expression in three fresh tumor samples (*a*, *b*, and *c*, lanes 3, 4, and 5, respectively) and in the same tumor samples after 12–16 days of culture with IL-7 (*a'*, *b'*, and *c'*, lanes 6, 7, and 8, respectively). Lane 9 (*d*) shows quantitative RT-PCR analysis of a tumor that was serially transplanted through 10 recipient mice. The positive control (+, lane 2) for these experiments was normal BM, and the negative control (–, lane 1) was BM in which reverse transcriptase was eliminated from the reactions.

splenocytes, tumor cells were found to be easily transplantable. Recipients generally became ill with widespread leukemia of donor origin (CD45.2) within 4–7 wk, and the tumors could be serially transplanted for at least 12 generations. The cell surface phenotype of transplanted tumor cells was essentially indistinguishable from the parental tumor, with the exception that the transferred cells showed a slight and reproducible down-regulation in the expression of Sca-1 (data not shown).

Tumor cells are IL-7 responsive

Because of the high level expression of B220 and CD43 on the leukemic cells, we were interested in determining the ability of these cells to grow and differentiate in response to the B cell cytokine IL-7, known to be essential for normal B cell development and in particular for the normal proliferation and differentiation of early pro-B cells (40). In the absence of cytokine, tumor cells remained viable for ~3–4 wk in primary in vitro culture and showed a low basal level of proliferation (Fig. 5A). After 5 days in culture with IL-7, the tumor cells proliferated rapidly (Fig. 5A) and maintained survival for up to 6 wk (data not shown). Interestingly, the cell surface phenotype of the cells changed following culture in IL-7. IL-7-treated cells remained B220^{bright}, CD43^{bright}, and IgM[–]; however, CD19 levels were up-regulated in the cultured cells and the cells lost expression of Gr-1 and Sca-1 (Fig. 5B). This was consistent with differentiation down the B lineage pathway in response to IL-7.

Gene expression and Ig rearrangements in IL-7-treated tumor cells

To investigate gene expression in these tumors, purified populations of leukemic cells were sorted from the spleen cell suspensions of three double Tg mice, followed by mRNA and genomic DNA isolation. Sorting diminished the possibility of significant contamination of the cell preparations with nonleukemic B cells. Each of the three tumor populations was also cultured in vitro with

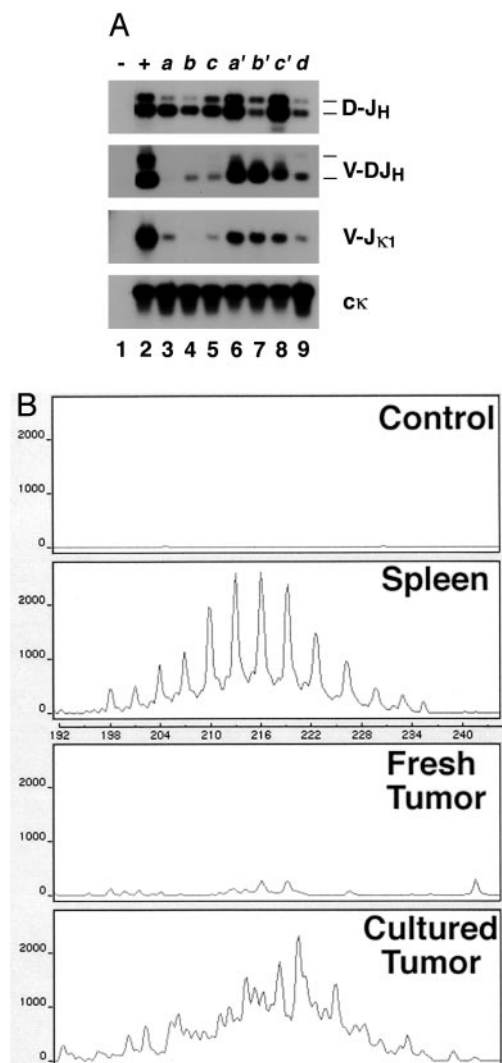


FIGURE 7. Induction of polyclonal Ig rearrangements in tumor cells cultured in IL-7. *A*, PCR assays to detect rearrangements at the H chain (D-J_H and V-DJ_H) and L chain (V-J_{κ1}) loci in three fresh tumor samples (*a*–*c*) and the same tumor samples after 12–16 days of culture with IL-7 (*a'*, *b'*, and *c'*). The last lane (*d*) shows RT-PCR analysis of a tumor that was serially transplanted through 10 recipient mice. The control PCR is a portion of the constant region in the κ locus (C κ). Positive control (+, lane 2), Normal BM; negative control (–, lane 1), BM, no reverse transcriptase in the reactions. *B*, V-DJ_{H2} joints were amplified from normal B6 spleen, fresh tumor cells, and IL-7-cultured tumor cells. Control in the upper panel is genomic DNA from mouse tail. The detected rearrangements in spleen shows a pattern of peaks separated by 3 bp, reflecting the selection for in-frame and productive V-DJ_{H2} joints. In contrast, the pattern in the cultured tumor cells does not show the triplets, indicating a lack of selection for productive joints, as expected.

IL-7 for 12–16 days before harvesting for mRNA and genomic DNA isolation and flow cytometric analysis. Semiquantitative reverse transcription and genomic PCR were then performed on the fresh and cultured samples, as well as on serially transplanted tumor cells at passage 10 (sorted based on B220, CD45.2, and CD43 staining). Cycle numbers were adjusted so that amplifications were in the linear range (generally between 22 and 30 cycles), and blotted PCR products were hybridized with radiolabeled internal oligonucleotides (36). The results of these analyses are shown in Figs. 6 and 7.

Fresh tumor cells (Fig. 6, *a*–*c*, lanes 3–5, respectively) expressed low levels of Rag2 and Rag1, TdT, the surrogate L chains

VpreB and $\lambda 5$, the B cell surface molecule CD19, and the B cell transcription factor BSAP. Each of these genes was induced following culture with IL-7 (Fig. 6, *a'*, *b'*, and *c'*, lanes 6–8, respectively). The transcription factor E47 and the Ig receptor-associated signaling molecule Ig β were expressed at equivalent levels in all samples. Sterile μ (μ°) transcripts, which indicate transcriptional activity at the unrearranged Ig H chain loci, were observed in fresh tumor samples and were down-regulated following culture in IL-7. Sterile κ (κ°) transcripts did not change. The RT-PCR results in the serially transplanted tumor sample (Fig. 6*d*, lane 9) generally mirrored the findings in the fresh tumor samples. Although low levels of CD19 mRNA were detected in fresh tumor, there were only background levels of surface CD19 staining by flow cytometry (see Fig. 5*B*).

The status of Ig H and L chain DNA rearrangements in the tumor cell populations was then examined by PCR of genomic DNA. D-J_H1 and D-J_H2 DNA rearrangements were identified at significant levels in both fresh and cultured cells (Fig. 7*A*, upper panel), while V(D)J and V-J κ 1 rearrangements were detected at low levels in the fresh tumor cells and were induced in cultured cells. Despite the evidence for Ig rearrangements in both fresh and cultured tumor cells, we were not able to demonstrate surface or intracellular expression of IgM (Fig. 5*B* and data not shown). This may reflect either a very low frequency of productive rearrangements or it remains possible that other genes required for the assembly and cell surface expression of IgM may not be expressed normally in these tumor cells.

The finding of rearrangements at both the J_H1 and J_H2 loci in the DJ_H and V(D)J assays (Fig. 7*A*) suggested the possibility that the tumors in these animals were polyclonal. To address the issue of clonality more definitively, we amplified genomic DNA from sorted fresh and cultured tumor cells with V-DJ_H2 primers and visualized the fluorescently labeled products on a denaturing sequencing gel. As shown in Fig. 7*B*, the V-DJ_H2 joints of tumor cells cultured with IL-7 were of multiple lengths, indicating the polyclonal nature of the tumors in double Tg mice.

Discussion

The data presented demonstrate that *bcl-x_L* can cooperate with *c-myc* in the malignant transformation of early B cell progenitors. Mice Tg for B cell-restricted *bcl-x_L* and *c-myc* transgenes developed highly malignant and fatal ALL. The median survival of double Tg animals was only 6 wk and no animals lived longer than 10 wk. Fresh tumor cells from these mice expressed the cell surface markers B220, CD43, Sca-1, Gr-1, Thy1.2, and CD4, a combination suggestive of a primitive stem cell origin (45). Interestingly, the tumor cells showed some evidence of commitment to the B lineage as evidenced by D-J_H Ig H chain gene rearrangements in freshly isolated tumor cells and the ability of the cells to differentiate down the B lineage in response to IL-7.

The *bcl-x_L* transgene used in these studies is driven by a combination of the μ enhancer and a herpes TK promoter (22). This Tg cassette drives high level expression of *bcl-x_L* early in B cell development, with much lower level expression in peripheral B cells. We also performed a series of experiments where we bred another B cell *bcl-x_L* Tg mouse (24) (transgene driven by μ enhancer and SV40 promoter giving higher expression in peripheral B cells than BM B cells), and characterized the resulting *bcl-x_L*-SV40/*myc* animals (data not shown). In previous "head-to-head" comparisons of the two Tg strains, it was demonstrated, for instance, that the *bcl-x_L*-SV40 transgene rescues *xid*-immunodeficient B cells from Ig-induced death while the *bcl-x_L*-Minnesota (MN) transgene does not (46), presumably due to the differences in timing and levels of transgene expression during B cell development.

It was of interest, then, that *bcl-x_L*-SV40/*myc* double Tg mice developed fatal tumors that were essentially undistinguishable from those in *bcl-x_L*-MN/*myc* mice (data not shown). The animals had a similar short life span, and fresh tumor cells showed an identical surface phenotype. *Bcl-x_L*-SV40/*myc* tumor cells also proliferated and progressed down the B lineage in response to IL-7 similar to *bcl-x_L*-MN/*myc* cells (data not shown). Thus, the two *bcl-x_L* transgenes were equally capable of synergizing with *myc* to induce transformation of an early B cell precursor cell, despite the fact that the *bcl-x_L*-SV40 transgene is likely expressed at significantly lower levels in early B cell precursors than the *bcl-x_L*-MN transgene.

One of the most striking features of the tumors that develop in *bcl-x_L*/*myc* or *bcl-2*/*myc* animals is the very primitive cell type that is targeted for malignant transformation. It is possible that the normal counterpart of these tumor cells is particularly susceptible to transformation by the combination of *myc* with either *bcl-x_L* or *bcl-2*. It is also possible that this early stage in B cell development is uniquely susceptible in the model because of the nature of the Tg expression constructs used to drive expression of the transgenes. Expression of both the *bcl-x_L* and *c-myc* transgenes is controlled by the Ig HC intronic enhancer, and the cell type targeted in these tumors is very close to the stage of B cell development where the HC intronic enhancer is first expressed at high levels. These tumors express a number of cell surface markers and genes (e.g., B220, CD19, sterile μ , and κ transcripts, Rag1, Ig β) characteristic of the earliest stages of pro-B cell development. The tumors in addition expressed Thy1.2, Gr-1, and Sca-1, consistent with a more primitive progenitor phenotype. This tumor cell phenotype may represent a low abundance population present in normal BM or the cells may express their unique combination of markers due to the effects of constitutive *myc* and *bcl-x* expression.

The tumors that arise in *bcl-x_L*/*myc* double Tg mice are similar in some ways to the tumors previously described in *bcl-2*/*myc* double Tg animals (17, 18). Fresh tumor cells from *bcl-x_L*/*myc* and *bcl-2*/*myc* animals have a similar cell surface phenotype. *Bcl-2*/*myc* tumors were B220⁺, CD43⁺, Sca-1⁺, Gr-1⁺, Thy-1⁺, and CD4⁺, virtually the same suite of markers expressed on *bcl-x_L*/*myc* cells. The time course of tumor progression in the two models was also similar, *bcl-2*/*myc* mice lived only ~6–8 wk.

The tumors that form in *bcl-x_L*/*myc* and *bcl-2*/*myc* mice also appear to differ in several significant ways. *Bcl-2*/*myc* tumors were reported to be very difficult to culture in vitro, with cells dying after only 1 or 2 days of in vitro culture (18). Although cell lines were eventually established from *bcl-2*/*myc* animals, their establishment required incubation with multiple cytokines along with growth on a stromal layer. In contrast, *bcl-x_L*/*myc* tumor cells have a relatively prolonged in vitro life span (days to weeks) and were highly responsive to a single cytokine (IL-7) in the absence of stromal support.

Several factors may contribute to the different phenotypes observed between the two models. This could reflect important functional differences between the *bcl-x_L* and *bcl-2* proteins during early B cell development, as suggested by the differing phenotypes of knockout animals lacking *bcl-x_L* or *bcl-2* (47, 48). *Bcl-x_L* knockout mice have very impaired early development of B cells, whereas *bcl-2* knockout mice show relatively normal initial development of B cells but then a dramatic loss of survival of naive splenic B cells. The knockout phenotypes are consistent with the idea that *bcl-x_L* may have a more important role in controlling survival early in B cell development than *bcl-2*.

The differing phenotypes of *bcl-x_L*/*myc* and *bcl-2*/*myc* tumor cells might also reflect differing availability of key regulatory

dimerization partners (such as *bax* and *bad*) in early B lymphoid progenitors and/or subtle differences in the stage of development in which the transgene is expressed due to the different Tg constructs used to drive expression. Finally, *bcl-x_L/myc* tumor cells, but not *bcl-2/myc* tumors, may be producing an autocrine cytokine (e.g., IL-7) or have other changes in gene expression that support improved survival or IL-7 responsiveness. Further studies will be required to establish the precise nature of these differences.

The IL 7 responsiveness of the *bcl-x_L/myc* tumor cells was impressive. IL-7 is known to be a critical and nonredundant growth and differentiation factor for mouse B lymphocytes, and mice with targeted deletions of the IL-7 gene or of the IL-7R α exhibit arrested B cell development at an early B cell stage of development (49). These data provide a striking example of how a tumor can retain cytokine responsiveness, which may contribute to its in vivo malignant behavior.

Recent data indicate that *bcl-x_L* expression is normally suppressed by *c-myc* in primary myeloid and pre-B cells (50). *Bcl-2* and *bcl-x_L* were also found to be markedly suppressed in the pre-cancerous cells of *E μ -myc* Tg B cells. Of interest, more than one-half of the spontaneous lymphomas that arise in *E μ -myc* Tg mice overexpress either *bcl-2* and/or *bcl-x_L* (50). The triggers for induction of *bcl-2* or *bcl-x_L* in this model system are not yet known. However, several well-described oncogenes (*Pim-1*, *c-myc*, *BCR-ABL*) are known to up-regulate *bcl-2*, and the NF- κ B pathway, which is turned on in many tumors, is a strong stimulus for *bcl-x_L* transcription (51). The ability of *myc* to drive cell proliferation and at the same time deliver a potent death signal may explain why antiapoptotic genes like *bcl-2* and *bcl-x_L* can synergize so potently with *myc* in malignant transformation.

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