**PIM1** gene cooperates with human **BCL6** gene to promote the development of lymphomas

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Diffuse large B-cell lymphomas in humans are associated with chromosomal rearrangements (~40%) and/or mutations disrupting autoregulation (~16%) involving the **BCL6** gene. Studies of lymphoma development in humans and mouse models have indicated that lymphomagenesis evolves through the accumulation of multiple genetic alterations. Based on our prior studies, which indicated that carcinogen-induced DNA mutations enhance the incidence of lymphomas in our mouse model expressing a human **BCL6** transgene, we hypothesized that mutated genes are likely to play an important cooperative role in **BCL6**-associated lymphoma development. We used retroviral insertion mutagenesis in an effort to identify which genes cooperate with **BCL6** in lymphomagenesis in our **BCL6** transgenic mice. We identified **PIM1** as the most frequently recurring cooperating gene in our murine **BCL6**-associated lymphomas (T- and B-cell types), and we observed elevated levels of **PIM1** mRNA and protein expression in these neoplasms. Further, immunohistochemical staining, which was performed in 20 randomly selected **BCL6**-positive human B- and T-cell lymphomas, revealed concurrent expression of **BCL6** and **PIM1** in these neoplasms. As **PIM1** encodes a serine/threonine kinase, **PIM1** kinase inhibition may be a promising therapy for **BCL6/PIM1**-positive human lymphomas.

Diffuse large B-cell lymphomas in humans are associated with a high mortality rate, and the majority of these contain mutations or chromosomal rearrangements that involve the **BCL6** gene (1). Prior studies of deregulated **BCL6** expression (2, 3) in mouse models have suggested that lymphoma development in these animals is hastened through the accumulation of multiple oncogenic hits. In an effort to define more targeted therapies for **BCL6**-associated lymphomas, we sought to identify which mutated genes cooperate with **BCL6** in lymphomagenesis. Here, through the use of retroviral insertion mutagenesis in our mouse model of the human **BCL6** transgene (2), we detected multiple retroviral insertion sites in the lymphomas (T- and B-cell types) in our animals, a phenomenon that resembles the mutational load noted in human B-cell lymphomas (4). We identified the gene called proviral integration site for Moloney murine leukemia virus 1 (**PIM1**) as the most frequently recurring cooperating gene in our murine **BCL6**-associated lymphomas. Immunohistochemical staining, which was performed in 20 randomly selected **BCL6**-positive human B- and T-cell lymphomas, revealed concurrent expression of **BCL6** and **PIM1**. Because **PIM1** encodes a serine/threonine kinase, our findings suggest that **PIM1** specific kinase inhibitors may be promising therapies for **BCL6/PIM1**-associated lymphomas.

**Results**

**MOL4070LTR Infection Accelerates Lymphomagenesis in **BCL6** Transgenic Mice.** Our **BCL6** transgenic mice are doubly transgenic animals, containing two transgenes: (i) human **BCL6** cDNA under control of the tetracycline-responsive minimal promoter (tet-o-**BCL6**) and (ii) the tetracycline-transactivating protein (tTA) under control of the Ig heavy-chain enhancer and the SRα promoter (EpiSR-tTA) (2, 5). As previously described (2), we generated two transgenic mouse lines, one containing four copies of the human **BCL6** transgene, the other 20 copies. We used the retrovirus MOL4070LTR (6) for retroviral insertional mutagenesis in our **BCL6** transgenic and control animals (nontransgenic mice of the same background). All animals were followed until natural death or necessity for euthanasia. It was possible to analyze the tissues from 92 of 99 transgenic mice injected with retrovirus and from 32 of 33 nontransgenic injected controls. Two animals in the transgenic group died on weaning, and tissues from all other excluded animals were too autolyzed to be analyzed.

Of 53 animals in the four-copy transgenic line, 24 (45.3%) developed lymphomas (18 T-cell, six B-cell; representative histology and flow data shown in Fig. 1). Of 39 mice in the 20-copy line, 10 (25.6%) developed lymphomas (three T-cell, seven B-cell). Lymphomas (**BCL6**-negative) also were anticipated in the control group, as the MOL4070LTR retrovirus is known to cause lymphomas and leukemias in mice (23% of FVB mice develop T-cell lymphomas by 270 d and approximately 50% of mice develop myeloid leukemias) (6). In our study, of 32 injected control (i.e., nontransgenic) animals, 11 (34.4%) developed lymphomas (five T-cell, six B-cell), with an unexpectedly high incidence of B-cell tumors compared with what is reported in the literature (6). Of 124 evaluable animals, 20 did not develop hematopoietic neoplasms (lymphoid or other, e.g., myeloid leukemias). Of these, three were in the control (i.e., nontransgenic) group and lived an average of 155 d (range 53–246 d), five were in the four-copy line and survived an average of 312 d (range, 254–392 d), and 12 were in the 20-copy line and lived an average of 249 d (range, 180–338 d).

We hypothesized that genes cooperating with **BCL6** in lymphomagenesis would be likely to promote faster lymphoma development in transgenic animals compared with control animals. Thus, for statistical purposes, we primarily focused on the first 180 d. Our four-copy **BCL6** transgenic line comprised 58% (53 of 92) of the analyzable mice in the study group, and the majority of the early lymphomas developed in this line (14 T-cell, two B-cell). Fig. 2 shows that the study (i.e., transgenic) group in this four-copy line had worse survival (hazard ratio, 3.8; 95% confidence interval, 1.1–13.0; P = 0.03, Cox regression) and, specifically, had a significantly worse 180-d lymphoma mortality rate (30.2%) than the control (i.e., nontransgenic) group (9.4%; P = 0.03, Fisher exact test).

The lymphomas usually were aggressive neoplasms. Most of the B-cell lymphomas were mature B-cell (7), CD19+, B220+, with associated splenomegaly (Fig. L4) and often lymphadenopathy. The majority of T-cell tumors (Fig. L8) could be...
Enhanced PIM1 RNA Expression in Lymphomas Containing Retroviral Insertions in or Near PIM1. Total RNA extracted from mouse lymphomas was reverse-transcribed and subjected to real-time quantitative RT-PCR. Each PCR yielded a single product. Lymphomas were reverse-transcribed and subjected to real-time quantitative RT-PCR. Each PCR yielded a single product. The lymphomas containing insertions in or near the PIM1 gene were identified in four female and three male mice, representing both of our BCL6 transgenic lines, ranging in age from 4 to 7.6 mo (average, 5.5 mo) at death. The viral integration site was 5′ of the PIM1 gene in two lymphomas, 3′ in three lymphomas, and within the fifth intron of the PIM1 gene in two additional cases (Table 1). One was a B-cell lymphoma (CD19+, B220+) with spread in multiple organs, and the others were T-cell tumors (precursor T-cell lymphoblastic lymphoma/leukemia per Bethesda classification system for murine lymphoid neoplasms) (7). They were aggressive malignancies, usually presenting as large thymic masses with spread in multiple organs.

Table 1. Relative PIM1 RNA in lymphomas from transgenic mice

<table>
<thead>
<tr>
<th>Type</th>
<th>Location of insert</th>
<th>Expression level vs. control</th>
</tr>
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<tbody>
<tr>
<td>T</td>
<td>Intron 5</td>
<td>63.3-fold</td>
</tr>
<tr>
<td>T</td>
<td>Intron 5</td>
<td>46.8</td>
</tr>
<tr>
<td>T</td>
<td>92.4 kb 5′ to PIM1</td>
<td>3.8</td>
</tr>
<tr>
<td>T</td>
<td>92.4 kb 5′ to PIM1</td>
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<td>T</td>
<td>4.4 kb 3′ to PIM1</td>
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<td>B</td>
<td>5.1 kb 3′ to PIM1</td>
<td>2.2</td>
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<tr>
<td>T</td>
<td>5.2 kb 3′ to PIM1</td>
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virus had inserted in or near the PIM1 gene in seven of 34 lymphomas (21%) in transgenic mice, but not in any of the 11 lymphomas that developed in the 32 control (i.e., nontransgenic) animals. Assuming that the retrovirus has an equal likelihood of inserting anywhere within the mouse genome (~25,000 genes), the finding that the lymphomas from seven transgenic mice (but no control animals) contained insertion sites within or near the PIM1 gene is highly statistically significant (P < 0.0001, binomial probability test).

The lymphomas containing insertion sites in or near the PIM1 gene were identified in four female and three male mice, representing both of our BCL6 transgenic lines, ranging in age from 4 to 7.6 mo (average, 5.5 mo) at death. The viral integration site was 5′ of the PIM1 gene in two lymphomas, 3′ in three lymphomas, and within the fifth intron of the PIM1 gene in two additional cases (Table 1). One was a B-cell lymphoma (CD19+, B220+) with spread to multiple organs, and the others were T-cell tumors (precursor T-cell lymphoblastic lymphoma/leukemia per Bethesda classification system for murine lymphoid neoplasms) (7). They were aggressive malignancies, usually presenting as large thymic masses with spread in multiple organs.

References:

Fig. 2. Lymphoma-related survival, BCL6 transgenic (i.e., four-copy) line. Cox regression and Fisher exact test show a significantly shorter latency to develop lymphoma for study (S; i.e., transgenic) mice (dashed line) compared with controls (C; i.e., nontransgenic) mice (solid line) at no more than 180 d; the 180-d lymphoma mortality rate was 9.4% in the control group and 30.2% in the study group (P = 0.03).
within the PIM1 gene had the highest PIM1 RNA levels (63.3- and 46.8-fold more, respectively, than the mean level found in the T-cell tumors from the nontransgenic retrovirus-injected mice; Table 1). These two insertion sites were within the fifth intron of the PIM1 gene (mouse chromosome 17; nucleotide positions 29,631,139 and 29,631,068, respectively). Table 1 shows that three lymphomas (one B-cell, two T-cell) contained inserts 3′ of PIM1, a region previously reported to be frequently affected by proviral integrations in murine T-cell lymphomas and associated with enhanced levels of PIM1 RNA (8). The only lymphoma that had a less than twofold elevation in PIM1 RNA level compared with the controls was a T-cell tumor with a 3′ viral insertion site.

**PIM1** **Protein Levels in Lymphomas from BCL6** **Transgenic Mice and Human** **BCL6-Positive Lymphomas.** PIM1 protein levels in murine B- and T-cell tumors detected by immunohistochemical staining correlated generally with PIM1 RNA levels from murine lymphomas quantified by real-time PCR (Fig. 4). As B5 expression interfered with anti-PIM1 staining, only formalin-fixed tissues were used. PIM1 protein was detectable in all of 20 randomly selected BCL6-positive human tumors (10 B-cell, 10 T-cell). PIM1 expression was variably cytoplasmic and/or nuclear. Nuclear expression, which is believed to correlate with PIM1 biologic effects (9), was detected at least focally in all the murine and human BCL6-positive lymphomas.

**Discussion**

Through the use of retroviral insertional mutagenesis in our unique human BCL6 transgenic mouse model, we identified PIM1 as a gene that cooperates with BCL6 to promote the development of lymphomas, and we showed the concurrent expression of the proteins encoded by these two genes in 20 randomly selected BCL6-positive human B- and T-cell lymphomas. It has long been known that BCL6 and PIM1 are both protooncogenes that play roles in the pathogenesis of lymphoma, but, in each case, supplemental factors (i.e., further oncogenic “hits”) are thought to be necessary for malignant transformation to take place (2, 3, 10). BCL6 expression is believed to become deregulated as a result of mutations that affect binding sites in the first (i.e., noncoding) exon (1) or chromosomal rearrangements in which normal BCL6 regulatory sequences are replaced by heterologous promoters (11). PIM1 has been reported as one of the multiple translocation partners that have been identified in rearrangements with BCL6 (12). PIM1 and BCL6 are associated with the somatic hypermutation events that occur in Ig genes (13, 14), a process that, when aberrant, is thought to contribute to malignant transformation (14); and both are expressed predominantly in lymphocytes (9, 15). In humans, overexpression of PIM1 has been noted in B-cell lymphomas (16) and has been associated with genomic instability (17) in prostate cells. However, in PIM1 transgenic mice, retroviral infection induces T-cell tumors (8). Even in PIM1 transgenic mice containing a leukemia virus long-terminal repeat supplemented with an upstream Ig enhancer and expressing high PIM1 RNA levels in both B and T cells, development of T-cell lymphomas was noted. Interestingly, these T-cell neoplasms were found to contain activated MYC (18), a gene usually associated with B-cell lymphomas in humans. Similarly, in MYC transgenic mice (5), and also in BCL6 (2) transgenic mice, predominantly T-cell lymphomas develop spontaneously (in the case of MYC) (5) or, in the case of BCL6, after induction with a retrovirus (as described here) or mutagen (2), even though these genes are associated predominantly with B-cell neoplasms in humans. Further, it has long been known that BCL6 is expressed not only in human B-cell neoplasms, but also in certain T-cell lymphomas (19), which are more recalcitrant to current treatment modalities than are B-cell tumors. Recently the role of BCL6 in T-cell development and function has received increasing attention (20–26). It is now believed that, even though BCL6 is not required for B or T-cell development, it is absolutely essential for germinal center B-cell (27, 28) and T follicular helper cell differentiation (20–22) and is important in other stages/subsets as well.

Human PIM1 encodes a 313-aa serine-threonine kinase that has antipapoptotic activity and is involved in cell survival, prolif-
eration, differentiation, transformation, tumor progression, as well as angiogenesis (29–31). Its protein is expressed in the thymus, liver, and spleen, and in hematopoietic progenitor cells in the bone marrow during development (32), as well as in the central nervous system (33). Although its cellular localization may be nuclear and/or cytoplasmic, nuclear localization is believed to be necessary for its biologic effects (9). In humans, PIM1 and c-MYC appear to have a cooperative role in prostate cancer (34), and increased expression of PIM1 has been noted in gastrointestinal cancers (35), pancreatic adenocarcinoma (36), and oral squamous-cell carcinoma (37). PIM1 also has been noted to be overexpressed in myeloid and lymphoid leukemias (38) and has been associated with B-cell lymphomas (4, 9). Because loss of PIM1 does not adversely affect the fertility or survival of KO mice (38), there has been intense recent interest in small molecules that inhibit PIM1 expression. SGI-1776, a small-molecule inhibitor of PIM1, PIM2, and PIM3 kinases, induced apoptosis in a human mantle cell lymphoma line and acute myeloid leukemia cell lines when studied as mouse xenografts and in acute myeloid leukemia cell lines in cell culture with little toxicity to normal human lymphocytes (39). This drug, which was in a phase I study in patients with refractory prostate cancer and lymphoma, was associated with cardiac toxicity, and further use in human trials was halted (http://clinicaltrials.gov/ct2/show/NCT00848001).

In conclusion, we have identified PIM1 as a cooperating gene with BCL6 in lymphomas that develop in retrovirus-injected mice expressing the human BCL6 transgene, and we have shown in our studies the concurrent expression of PIM1 and BCL6 in a limited number of randomly selected BCL6-positive human lymphomas. Although PIM1 has long been known to be a protooncogene, its association with BCL6 has not been described to our knowledge. It would be of interest to analyze further the cooperation between PIM1 and BCL6 by breeding Eq-pim-1 transgenic mice (18) with our mice transgenic for human BCL6 (2) and with the engineered mice of Cattoretti et al. (3). We suggest that study of human lymphomas for PIM1 expression (which is not currently a part of the standard diagnostic lymphoma assessment panel) be added to the routine testing that is currently performed on human B- and T-cell tumors. Our results imply that inhibition of PIM1 kinase in human BCL6-positive and/or PIM1-positive lymphomas may be a useful therapeutic modality in this disease.

Materials and Methods

Retroviral Insertional Mutagenesis. Under approved institutional protocols, we injected 99 B6C3F1 transgenic mice (2) and 33 nontransgenic controls (animals of the same background, WT or positive for either (but not both) the 6.3-tTA or BCL6 transgenes) (2) with MOL4070LTR recombinant murine retrovirus (8), 10^5 infectious units intraperitoneally 1 d after birth, and followed them until euthanasia or death, at which time all animals underwent necropsy.

Histology and Flow Cytometry. Tissues were fixed in 10% (vol/vol) buffered formalin, paraffin-embedded, sectioned, stained with H&E, and studied with an experienced hematopathologist who was blinded whether the mouse was transgenic or control. Hematologic malignancies were analyzed by FACS whenever possible. Single-cell suspensions were incubated for 15 min with anti-2.4G2 to inhibit nonspecific binding, then labeled with fluorochrome-conjugated antibodies (BD Biosciences/Pharmingen or Sigma-Aldrich) according to the manufacturer’s protocol. Analysis was performed on an LSRII flow cytometer (BD Biosciences). Markers included antibodies directed against murine B and T cells, macrophages, myeloid and erythroid cells, immature cells (e.g., Sca-1, CD117), immunoglobulins, κ-, λ-, and germinal center cells (e.g., peanut agglutinin). Lymphoid neoplasms were classified according to the Bethesda proposals (7).

Inverse PCR and Database Searches. DNA from all analyzable lymphomas was purified and subjected to inverse PCR as described (40), except that 29 cycles of PCR amplification were performed in a 20-μL final volume, with annealing at 61 °C for the 5′ LTR primers and 56 °C for the 3′ LTR primers, and the final extension step was 15 min. Pools of PCR products were cloned and transformed with a TOPO TA Cloning Kit for sequencing (Invitrogen). Purified DNA from ampicillin-resistant colonies was sequenced with a Big Dye Terminator cycle sequencing kit (v3.1; Applied Biosystems) on a 3730xl DNA Analyzer (Applied Biosystems) by using primers from the pCR4-TOPO vector (Invitrogen) in which the inserts had been cloned. Sequences were matched to murine databases of the University of California Santa Cruz (genomic build of July 2007) genome browser and the National Institutes of Health. As it is known that gene expression can be affected over hundreds of kilobases by retroviral integrations (41) and chromosomal translocations (42), we studied potentially affected genes within at least a 100-kb window of an insertion site. Candidate genes identified in this way were considered as possibly relevant if they were noted in at least two transgenic mice but not in any controls (43, 44).

Real-Time Quantitative PCR. Total RNA was extracted from mouse lymphomas, DNase-I-digested, reverse transcribed, and subjected to real-time RT-PCR (along with “no-RT” controls) with the use of SYBR Green Master Mix (Takara Bio) according to the manufacturer’s instructions and a LightCycler 480 (Roche Applied Science). Primers included β-actin (Promega) that amplifies 285 bp (2) of cDNA (vs. 396 bp for genomic DNA) and primers that amplify 176 bp of cDNA of the murine PIM1 gene (5′ primer in exon 5, 3′ primer in exon 6; Real Time Primers). Controls included water template and RNA extracted from randomly selected lymphomas (three T-cell and three B-cell lymphomas) of the comparable organs of retrovirus-injected nontransgenic mice. Results from triplicate wells (for each study animal) or duplicate wells (multiple control mice) were averaged and compared.

Immunohistochemistry. Human lymphoma paraffin-embedded blocks were obtained from the surgical pathology archives under an institutional review board-approved protocol. Immunohistochemical staining on human tissues was performed with mouse monoclonal anti-human BCL6 (clone LN22; Novoceastra) at a dilution of 1:20 on the automated Bond system (Leica Biosystems) with Bond Polymer Refine Detection. Antigen retrieval was performed in Epitope Retrieval Solution 2 for 20 min (Leica Microsystems). A modified protocol included antibody incubation for 25 min, post-primary reagent 15 min, polymer 25 min, peroxidase block 8 min, 3,3′-diaminobenzidine 10 min, and hematoxylin 8 min. BCL6 staining on mouse tissues was performed as described previously (2). Additionally, paraffin-embedded formalin-fixed human and murine lymphomas were stained with anti-PIM1 (purified rabbit polyclonal antibody, AP7932d at dilution 1:50; Abgent). For anti-PIM1 staining, antigen retrieval was performed in Bond Epitope Retrieval Solution 2 for 20 min; the peroxidase block, post-primary rabbit anti-mouse IgG, and hematoxylin applications were omitted, and the antibody was applied twice (25 min each). Images were taken with a BX41 microscope (Olympus), a DP72 digital camera, and cellSens Standard imaging software (Olympus).

Statistical Analysis. Lymphoma-related survival in the study vs. control mice was compared with the use of Cox regression and Fisher exact test (to look further at lymphoma-related survival at specific time points). Kaplan–Meier curves also were generated for each group. The data from real-time PCR in the study and control groups were analyzed by the comparative Ct method (45) and compared by the Wilcoxon rank-sum test. Statistical analyses were performed by using Stata software (version 12; StataCorp).

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