Transgenic mice overexpressing in B lymphocytes either Bcl-2 or a TNF receptor-associated factor (TRAF)2 mutant lacking the N-terminal RING and zinc finger domains located at the N terminus of the molecule (TRAF2DN), which mimics TRAF1, developed lymphadenopathy and splenomegaly due to polyclonal B cell expansion. Remarkably, TRAF2DN/Bcl-2 double-transgenic mice contained B cell populations similar to those observed in TRAF2DN mice. However, over time, they developed severe splenomegaly and lymphadenopathy, and most animals also developed leukemia, pleural effusion, and, in some cases, ascites associated with monoclonal adenopathy, as a result of a polyclonal expansion of B lymphocytes (14). Interestingly, TRAF2DN is structurally similar to TRAF1, which is the only TRAF family member that lacks a RING finger domain (6). TRAF1 and TRAF2DN can hetero- dimerize with TRAF2, modulating various TRAF2 activities (6, 10, 14, 15).

In this report, we show that transgenic mice expressing both TRAF2DN and Bcl-2 in the B cell lineage develop an age-dependent B cell leukemia and lymphoma having striking similarities to human CLL. These findings provide direct evidence that TRAFs can contribute to malignancy. Furthermore, our results provide functional evidence that the high levels of TRAF1 and Bcl-2 coexpression commonly found in human CLL cells contribute to the pathogenesis of this leukemia.

Materials and Methods

Transgenic Mice. Transgenic BALB/c mice expressing human Bcl-2 specifically in B lymphocytes have been described (12). The transgene mimics the (14, 18)(q32;21) translocation involving Bcl-2 and IgH found in human follicular lymphomas (FLs) (Fig. 1A). Transgenic FVB/N mice expressing a TRAF2 mutant lacking the N-terminal 240 amino acids encompassing the RING and zinc finger domains (TRAF2DN) have been described (14). Bcl-2 and TRAF2DN heterozygous mice were bred to produce litters with progeny of all four genotypes (wild-type, Bcl-2 single-positive, TRAF2DN single-positive, and TRAF2DN/Bcl-2 double-positive mice) expressed on mixed BALB/c × FVB/N. Analysis of the transgenic mouse genotypes was performed by PCR by using primers specific for human Bcl-2 and TRAF2DN, and verification of the transgene expression was accomplished by immunoblotting. Euthanasia

Abbreviations: TNFR, TNF family receptor; TRAF, TNF-receptor-associated factor; CLL, chronic lymphocytic leukemia; PI, propidium iodide; TRAF2DN, TRAF2 mutant lacking the RING and zinc finger domains located at the N terminus of the molecule; APC, allophycocyanin; PE, phycoerythrin; IgH, Ig heavy chain; FL, follicular lymphoma.

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was performed by following the rules of the American Veterinary Medical Association.

**Cell Isolation.** Spleens and lungs were carefully crushed to release the lymphocytes. Ascitic fluid was obtained from the peritoneal cavity, and blood was collected in tubes coated with heparin from the heart of euthanized mice or from the cavernous sinus. Cell suspensions were depleted of red cells and neutrophils by density centrifugation (Lympholite-M, Cedarlane Laboratories) or by hypotonic lysis of erythrocytes. Then, for cell quantification, blood was collected in heparinized capillary tubes, diluted five times in PBS containing 10% FCS, 1% BSA, 0.05% sodium azide, 5 mM EDTA, and 50 μg/ml human γ-globulin, and incubated on ice for 15 min. Then the mixture was incubated with anti-CD45 PE-Cy5 and anti-B220 PE or the respective isotype controls and incubated for 20 min at room temperature. Erythrocytes were lysed by using 10 volumes of hypotonic lysis buffer (PharmLyse, BD Biosciences), and quantification was performed on a personal cell analysis and counter microfluorocytometer (Guava Technologies, Hayward, CA).

For DNA content analysis, cells were washed and resuspended in 0.4 ml of hypotonic buffer containing 0.1% sodium citrate, 0.37% Nonidet P-40, 20 μg/ml RNase A, and 50 μg/ml PI. Cells were incubated for 5 min on ice and analyzed by flow cytometry.

**Immunohistochemistry.** Tissues and organs from transgenic mice were fixed in Bouin’s solution (Sigma) and embedded in paraffin, and tissue sections (5 μm) were stained with hematoxylin/eosin and for immunohistochemistry as described (7, 16). Bone marrow, blood smears, and ascites were stained with Wright–Giemsa staining.

**Immunoblotting.** Cell lysates from frozen mouse lymphoid tissues were prepared in modified Laemmli buffer, as described (17). Tissue lysates were normalized for total protein content (50 μg per lane) and subjected to SDS/PAGE followed by immunoblot analysis using anti-human Bcl-2 (16), anti-mouse Bcl-2 (18), anti-TRAF2 (Santa Cruz Biotechnology, C-20), and anti-Tcl-1 (Santa Cruz Biotechnology, F-14) Abs. Detection was accomplished by using appropriate secondary horseradish peroxidase-conjugated Abs (Bio-Rad), followed by an enhanced chemiluminescence assay (Amersham Pharmacia).

**Southern Blotting.** Ig heavy chain (IgH) gene rearrangements were analyzed by Southern blotting by using a mouse IgH probe that spans J_{H}2 and E_{μ}, essentially as described (20).

**In Vitro Cell Survival Assays.** Purified splenocytes (5 × 10^{5} cells per well) were cultured in RPMI medium 1640 containing 10% FCS (HyClone), 50 μM 2-mercaptoethanol, oxalate, pyruvate and insulin supplement (Sigma), 1 mM L-glutamine, and antibiotics in the presence of different chemotherapeutic drugs. Cells were recovered by centrifugation at various times and stained with APC-anti-B220 mAb, and cell death of the B220^+ lymphocytes was determined by double staining with FITC-labeled annexin-V and PI (BioVision, Mountain View, CA), followed by fluorescence-activated cell sorter analysis using the FL-1 and FL-3 channels. Annexin-V-positive cells were considered apoptotic.

**Results**

**Mice Expressing TRAF2DN and Bcl-2 Develop Massive Splenomegaly and Die Prematurely.** Many B cell malignancies, including CLL and FL, are characterized by excessive Bcl-2 expression, which is important to the etiology of these diseases (3). In addition, increased levels of TRAF1 expression have also been observed in many CLls and FLs (6, 7). However, the relevance to B cell malignancy of combined up-regulation of Bcl-2 and TRAF1 has
mice (H11005) remained alive (Fig. 1) after birth. By 14 months, reduced survival. Some of these mice died as early as 6 months.

B different genotypes (Fig. 1) immunoblotting by using spleens from age-matched mice of the implicated in development of CLL and lymphoma (21, 22), were reduced in wild-type and Bcl-2 transgenic littermates (Fig. 1). Accordingly, expression of the lung Krüppel-like factor, impairment of TRAF2 activities in the TRAF2DN transgenic TRAF2 protein. The mechanism of TRAF2DN-mediated down-regulation of TRAF2 is not yet known, but it further implies an impairment of TRAF2 activities in the TRAF2DN transgenic mice. Accordingly, expression of the lung Krüppel-like factor, which depends on TRAF2 (11), is severely reduced in TRAF2DN and TRAF2DN/Bcl-2 splenocytes compared with wild-type and Bcl-2 transgenic littermates (Fig. 1B). In contrast, similar levels of T cell lymphoma (TCL)-1 protein, previously implicated in development of CLL and lymphoma (21, 22), were found in all mouse lines.

TRAF2DN/Bcl-2 Double-Transgenic Mice Develop Small B Cell Lymphoma and CLL-Like Leukemia. Histological analysis of TRAF2DN and TRAF2DN/Bcl-2 spleens consistently showed the expansion of the marginal zone with infiltrating small B lymphocytes (Fig. 2). In contrast, Bcl-2 mice also had an expanded population of small B cells consisting of centrocytes that typically expanded the splenic white pulp (not shown).

Immunohistochemical analysis of TRAF2DN/Bcl-2 mice with overt disease showed massive infiltration of B220-positive lymphocytes throughout major organs and tissues (Fig. 2). TRAF2DN single-transgenic mice also showed lymphocyte infiltration in lungs, but never to the extent of TRAF2DN/Bcl-2 double-transgenic mice (data not shown). The bone marrow of mice with overt disease was massively infiltrated with B220-positive small lymphocytes, which would be consistent with the development of leukemia (see below).

We characterized the lymphoid cell populations from these mice by fluorescence-activated cell sorter (FACS) analysis. The B cells found in Bcl-2 mice are similar to those found in wild-type mice, which are mainly mature B cells expressing B220+/IgM+LO/IgDHI/CD210/CD230/CD5null (Fig. 3). TRAF2DN mice have an expanded subpopulation of B220+/CD210/CD230 B cells. (Fig. 3). Young or asymptomatic TRAF2DN/Bcl-2 mice contained expanded B cell populations similar to those found in the TRAF2DN single-transgenic mice (Fig. 3). In contrast, FACS analysis of splenocytes from TRAF2DN/Bcl-2 mice that died naturally or that had developed leukemia consistently showed a dramatic expansion of a population of B220+/IgM+IQGDI:to null/CD210:to null/CD23null/CD11b+ B cells. In the majority of the mice, these cells also expressed CD5, consistent with a B-1a phenotype. In one-quarter of the mice, this cell population did not express CD5, consistent with a B-1b phenotype (26) (Fig. 3 and Figs. 7 and 8, which are published as supporting information on the PNAS web site). These mice also developed leukemia, with B cell counts in blood as high as 167 × 10⁶ cells per ml, compared with an average for wild-type mice of 4 × 10⁶ B cells per ml. The accumulation of leukemic B cells in blood over a period of 3 weeks is shown in Fig. 9A, which is published as supporting information on the PNAS web site. Analysis of the blood B lymphocytes of leukemic mice consistently shows the expansion of the B-1-like B220+/IgM+IgDI:tonull/CD21:tonull/CD23null B cells (Fig. 3 and Fig. 9B, which is published as supporting information on the PNAS web site).

As indicated above, we also found this B-1-like population in...
phenotypically and morphologically similar in many respects to (SBL) with a leukemic phase (25). This mouse SBL is immuno-
thus fully consistent with the diagnosis of small B cell lymphoma
stage and splenocytes or blood lymphocytes of a representative TRAF2DN
Bcl-2 double-transgenic mice at a premalignant
cytos were analyzed from representative 11-month-old wild-type, Bcl-2,
CD5 and isotype control analyses. Spleno-
ymphocyte population for B220
Bcl-2 mouse (12 months old) with acute disease.

B cell expansions observed in the TRAF2DN
populations of the TRAF2DN
alterations caused by the microenvironment.

Increased Expression of Adhesion Molecules on the TRAF2DN/Bcl-2 B Lymphocytes. Increased expression of cell adhesion molecules has been implicated in invasiveness of malignant B cells (26). Because TRAF2DN/Bcl-2 double-transgenic mice developed massive lymphoid infiltration in nonlymphoid tissues (Fig. 2), we analyzed the expression of various cell adhesion molecules on the surface of B lymphocytes from transgenic mice, including CD54 (intercellular adhesion molecule-1) and CD29 (b1 integrin) (Fig. 4). B cells from wild-type and Bcl-2 transgenic mice had comparable CD54 and CD29 expression levels. However, B cells
from TRAF2DN and asymptomatic TRAF2DN/Bcl-2 mice showed increased expression of CD54 and CD29 (Fig. 4A). This increase in CD54 and CD29 expression was even more evident in B cells from TRAF2DN/Bcl-2 mice in the acute phase of the disease ($P < 0.05$) (Fig. 4 B and C). The B cells with high CD54 and CD29 are those expressing B220$^{+}$/IgM$^{+}$/IgD$^{L}$tonull/C
CD21Ltonull/CD23$^{null}$ whereas normal mature B cells expressing B220$^{+}$/IgM$^{+}$/IgD$^{H}$/CD21$^{+}$/CD23$^{H}$ bear normal levels of CD54 and CD29 (Fig. 9B). Analysis of the expression of CD11a (LFA-1) and CD49d (a4 integrin) also revealed a moderate increase in the expression of these adhesion proteins on B cells in TRAF2DN and TRAF2DN/Bcl-2 mice (not shown).

Analysis of Ig Gene Rearrangements in B Cells from the TRAF2DN/Bcl-2 Double-Transgenic Mice Reveals Clonal Neoplasms. The increase in B cell numbers seen in TRAF2DN/Bcl-2 mice upon progression to the symptomatic phase could result from either the outgrowth of neoplastic B cell clones or a gradual unrelenting accumulation of polyclonal B cells. To distinguish between these possibilities, we analyzed DNA from splenocytes and pleural effusion of transgenic mice for the presence of clonal Ig gene rearrange-
ments. As shown in Fig. 5, the existence of a single J$eta$ rearrangement (mice 83, 148, 316, 302, and 327) is indicative of the
expansion of a single B cell clone in the spleens of these TRAF2DN/Bcl-2 mice that progressed to overt disease, whereas multiple rearrangements were observed in mouse 137, indicative of the expansion of different clones. In contrast, no evidence of clonality was observed in Bcl-2 (data not shown), TRAF2DN, or asymptomatic (premalignant) TRAF2DN/Bcl-2 mice (Fig. 5).

Apoptosis Resistance of TRAF2DN/Bcl-2 B Cells. The expansion of B cells in the double-transgenic mice could have occurred because of accelerated cell division, reduced cell death, or both. To explore the basis for the expansion, we performed cell proliferation assays of
both nodal and extranodal locations. However, some terminally ill mice also had expansions of phenotypically different B lymphocyte subpopulations, especially in ascitic fluid or lung compared with spleen or blood of the same mouse (Fig. 8). This population heterogeneity might be caused by oligoclonal expansion of malignant lymphocytes or could reflect phenotypic alterations caused by the microenvironment.

Bel-6 expression was not detected in the expanded B cell populations of the TRAF2DN/Bcl-2 double-transgenic mice, thus confirming a pregerminal center origin (24) (data not shown). Total numbers of T cells (CD3 positive) as well as the
CD4/CD8 ratio were similar in wild-type and transgenic mice
(data not shown).

The combined histopathologic and phenotypic features of the B cell expansions observed in the TRAF2DN/Bcl-2 mice are thus fully consistent with the diagnosis of small B cell lymphoma (SBL) with a leukemic phase (25). This mouse SBL is immunophenotypically and morphologically similar in many respects to human CLL.
lymphocytes isolated from the various transgenic mice. As shown in Fig. 6A, cells from Bcl-2, TRAF2DN, and asymptomatic TRAF2DN/Bcl-2 double-transgenic mice had proliferation rates comparable to those of cells from wild-type littermates (Fig. 6A). Consistent with these results, no increase in the number of cells over a period of 2 weeks was observed in cultures of TRAF2DN/Bcl-2 splenocytes, asces, or blood lymphocytes isolated from mice with leukemia and overt lymphoma (n = 4). DNA content analyses were then performed to assess the cell cycle distribution of lymphocytes isolated from TRAF2DN/Bcl-2 double-transgenic mice that developed overt lymphoma. The percentage of cycling cells as evidenced by DNA content >2n (i.e., cells in S, G2, or M phase) was only slightly higher in splenocytes of TRAF2DN/Bcl-2 double-transgenic mice with overt lymphoma (9.1 ± 2%), compared with age-matching wild-type mice (6.3 ± 0.9; P > 0.2), Bcl-2 single-(6.2 ± 0.2%), and TRAF2DN single-transgenic mice (5.6 ± 0.6%) (Fig. 6B a–d). However, these differences were not observed in lymphocytes isolated from ascitic fluid or extracted from lungs of symptomatic TRAF2DN/Bcl-2 double-transgenic mice (5.3 ± 0.5% and 5.8 ± 0.7%, respectively) (Fig. 6B d and e). Furthermore, immunostaining of lymphoid organs of TRAF2DN/Bcl-2 mice with Ab to proliferating cell nuclear antigen (PCNA) revealed only occasional (<6%) PCNA-positive cells before and after progression to overt disease (data not shown).

We next tested whether B cells from the transgenic mouse lines displayed resistance to apoptosis. Splenocytes from the TRAF2DN single-transgenic mice and wild-type littermates had similar spontaneous apoptosis rates after 24 h in culture (36.2 ± 10.8% and 34.6 ± 6.2%, respectively). In contrast, the apoptosis rates were significantly reduced in Bcl-2 single- and TRAF2DN/Bcl-2 double-transgenic mice (14.7 ± 7.5 and 18.7 ± 9.4%, respectively) (Fig. 6C).

Lymphocytes from the Bcl-2, TRAF2DN, and TRAF2DN/Bcl-2 double-positive mice were also tested for apoptosis induced by chemotherapeutic drugs commonly used in the treatment of CLL, including the nucleoside analog fludarabine and the glucocorticoid dexamethasone (Fig. 6D). B cells from the Bcl-2 and TRAF2DN/Bcl-2 double-transgenic mice displayed marked resistance to apoptosis induced by these drugs, particularly to dexamethasone (Fig. 6D). Interestingly, B cells from the TRAF2DN single-transgenic mice also displayed significant resistance to both dexamethasone- and fludarabine-induced apoptosis (Fig. 6D), thus suggesting that both TRAF2DN and Bcl-2 protect B cells against apoptosis.

Altogether, these results strongly suggest that resistance to apoptosis rather than deregulation of proliferation accounts for the malignant transformation of lymphocytes in TRAF2DN/Bcl-2 double-transgenic mice.

Discussion

The results described here show that the combined deregulated expression of Bcl-2 and TRAF2 resulted in development of small B cell lymphoma/CLL in mice. Our criteria for defining this disease as a CLL-like disorder are as follows: (i) the mice develop leukemia; (ii) the neoplastic B cells express CD5; (iii) they do not express Bcl-6; (iv) they progress to clonal malignancies that are lethal; (v) the cells are not rapidly proliferating; and (vi) the B cells display an apoptosis-resistant phenotype.

The data presented above suggest that a blockage to apoptosis with subsequent additional secondary genetic lesions resulting in
further growth advantage is the cause of the malignant B cell expansion that occurs during disease progression in the TRAF2DN/Bcl-2 mice. In this regard, some lines of transgenic mice overexpressing Bcl-2 in B cells develop plasmacytoma and pre-B cell lymphoma (13), although the frequency of lethal malignancy is generally low (only 3–15% of the mice). However, the Bcl-2 transgenic line we used (12) did not develop tumors and had a normal life span, despite polyclonal expansion of B cells. These results indicate that Bcl-2 alone is not sufficient for malignant transformation, and that additional factors are required for development of B cell malignancy. An example of a cooperating gene is c-MYC, which collaborates with Bcl-2 to induce aggressive lymphomas (27). However, those lymphomas are comprised of large cells (diffuse histiocytic lymphocytes) different from the small B cell lymphoma that arises in TRAF2DN/Bcl-2 mice.

Our data revealed a role for TRAF-family proteins in tumorigenesis. TRAFs integrate signals from many members of the TNFR family and regulate multiple cellular processes, including proliferation, gene expression, and apoptosis. However, until now, no evidence has been reported supporting direct involvement of TRAF-family proteins in cancer. TRAF2 is a prototypical member of the TRAF family, comprised of a RING finger domain within its N terminus, followed by five zinc finger domains and the TRAF domain (5, 6). TRAF2DN is similar to TRAF1, and both proteins are known to bind many of the same proteins via their TRAF domains (10, 14). The RING and finger domains are essential for many TRAF2 activities, such as Jun N-terminal kinase activation and downstream signaling molecules. Interestingly, we observed that TRAF2DN expression in B cells also resulted in a marked decrease in endogenous TRAF2 protein, further supporting a role for TRAF2DN as a TRAF2 antagonist in our system. In humans, TRAF1 is overexpressed in many CLL and FLs. In CLL, TRAF1 expression is associated with more aggressive chemoresistant disease (7). Whether TRAF1 confers a survival advantage to CLL is unclear at present. However, our evidence of an antiapoptotic role for TRAF2DN in B cells suggests that TRAF1 may have a similar function. The mechanisms underlying the antiapoptotic effects of TRAF1 and TRAF2DN in B lymphocytes could involve (i) effects on Jun N-terminal kinase, which plays an essential role in TNF-induced apoptosis (28); (ii) effects on NFκB activation, which is required for resistance to apoptosis in some circumstances (29); and (iii) inhibition of signaling by TNF-family death receptors, thus complementing the Bcl-2-mediated suppression of the mitochondrial pathway for apoptosis.

The B cells that accumulate in the spleen and nodes of TRAF2DN single- and TRAF2DN/Bcl-2 double-transgenic mice express elevated surface levels of adhesion proteins. Thus, TRAF2DN may modulate cell invasiveness through up-regulation of cell adhesion proteins such as CD54. However, despite increased expression of adhesion proteins, B cells of TRAF2DN single-transgenic mice did not accumulate in extranodal tissues to nearly the extent observed in TRAF2DN/Bcl-2 mice. We speculate, therefore, that once leaving the nodal compartment, TRAF2DN B cells die unless apoptosis is blocked by Bcl-2. In this scenario, Bcl-2 overexpression would allow the survival of those B cells that have migrated into extranodal environments, as has been suggested for other members of the Bcl-2 family (30). We also suggest that the ability of TRAF2DN/Bcl-2 to accumulate in extranodal tissues is a manifestation of the complementation of the abilities of Bcl-2 to promote survival in ectopic sites and TRAF2DN to promote invasiveness. It remains to be determined which genetic events contribute to progression of TRAF2DN/Bcl-2 B cells to monoclonal malignancies followed by induction of a leukemic phase and death.

We thank Dr. J. Ward (Veterinary and Tumor Pathology Section, National Cancer Institute, Frederick, MD) for expert evaluation of tissue sections; Dr. W. Plunkett (MD Anderson Cancer Center, Houston) for F-ara-A; Dr. S. Janz (Center for Cancer Research, National Cancer Institute, Bethesda) for the pJ1 IGH probe; Dr. L. Glimcher (Harvard Medical School, Boston) for the anti-MaK/CD21 antibody; and C. L. Kress, M. Thomas, I. M. Pedersen, M. Mcalonis, X. Xiao, C. Yost, L. Fiorentino, and the personnel of the animal facility at the Burnham Institute for excellent technical assistance. We also thank Dr. S. Krajewski, members of the CLL Research Consortium and Guava Technologies (Hayward, CA) for helpful discussions and advice. This study was supported by National Cancer Institute Grants CA9381 and CA81534.