Dysregulated TCL1 requires the germinal center and genome instability for mature B-cell transformation

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Most lymphomas arise by transformation of germinal center (GC) B cells. *TCL1*, a proto-oncogene first recognized for its role in T-cell transformation, also induces GC B-cell malignancies when dysregulated in *pEμ-B29-TCL1* transgenic (TCL1-tg) mice. Clonal B-cell lymphomas develop from polyclonal populations with latencies of 4 months or more, suggesting that secondary genetic events are required for full transformation. The goals of this study were to determine the GC-related effects of TCL1 dysregulation that contribute to tumor initiation and to identify companion genetic alterations in tumors that function in disease progres- **sion. We report that compared with wild-type (WT) cells, B cells from TCL1-tg mice activated in a manner resembling a T-dependent GC reaction show enhanced resistance to FAS-mediated apoptosis with CD40 stimulation, independent of a B-cell antigen receptor (BCR) rescue signal. Mitogenic stimulation of TCL1-tg B cells also resulted in increased expression of *Aicda*. These GC-related enhancements in survival and *Aicda* expression could underlie B-cell transformation. Supporting this notion, no B-cell lymphomas developed for 20 months when TCL1-tg mice were crossed onto an Oct coactivator from B cell (OCA-B)–deficient back-** **ground to yield mice incapable of forming GCs. Spectral karyotype analyses showed that GC lymphomas from TCL1-tg mice exhibit recurrent chromosome translocations and trisomy 15, with corresponding MYC overexpression. We conclude that *pEμ-B29-TCL1* transgenic B cells primed for transformation must experience the GC environment and, for at least some, develop genome instability to become fully malignant. (Blood. 2006; 108:1991-1998)**

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Introduction

Most common B-cell lineage lymphomas in humans originate by transformation of germinal center (GC) B cells. These include classes of non-Hodgkin lymphoma (NHL) designated follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), and Burkitt lymphoma (BL) as well as classical Hodgkin lymphoma.[1] FL and BL are characterized by recurring reciprocal chromosome (Chr) translocations that fuse *Ig* genes either with the *BCL2* cell survival gene or with the *MYC* proto-oncogene, respectively.[2,3] Also, reciprocal Chr translocations involving the *BCL6* transcriptional repressor gene and more than 20 partner loci, including the *Ig* genes, typify DLBCL.[4] These and additional tumor-promoting genes are subsequently expressed under the control of *Ig* regulatory sequences rather than their native regulatory elements, resulting in dysregulated expression. Interestingly, *MYC*, *BCL6*, and other genes, such as *TCL1*, are also expressed at high levels in many NHLs in the absence of activating translocations. In these cases, inappropriately high expression is a consequence of other genetic or possibly epigenetic alterations and is likely contributory to the transformation process.[5-7]

The *TCL1* proto-oncogene encodes a 14-kDa intracellular protein that associates in a multimeric complex with the serine/threonine kinase AKT.[7-11] Interactions with TCL1 enhance AKT activation and, by an unknown mechanism, TCL1 stimulates the protein kinase C–mitogen-activated protein kinase–extracellular signal–related kinase (PKC-MAPK-ERK) signal transduction pathway, promoting cell survival and proliferation.[8,12,13] In human T cells, *TCL1* is normally expressed only by immature cortical thymocytes and by activated peripheral T cells.[13,14] However, dysregulated expression from Chr rearrangements between *TCL1* and T-cell receptor *(TCR)* loci results in persistent high-level expression in mature T-cell leukemia/lymphomas.[15] A direct, initiating role for heightened *TCL1* expression in T-cell transformation is suggested by studies of transgenic mice with T-cell lineage–restricted *TCL1* expression that develop mature T-cell leukemias following an extended premalignant phase of polyclonal expansion.[16]

Despite its original identification in T-cell leukemias, *TCL1* seems to have an even more prominent role in B cells and is

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differentially expressed during normal B-cell maturation. Expression is robust from pre-B through follicular B-cell development, but is down-regulated during the GC reaction and is silenced in post-GC memory B and plasma cells.[17] High levels of *TCL1* expression have been documented for human B-cell lymphomas originating from pre-GC and GC B cells and in AIDS-related lymphomas of post-GC cell origin.[17-19] The suggestion that inappropriately high expression in the GC could contribute to GC B-cell lymphomagenesis is strongly supported by studies of mice bearing a *pEμ-B29-TCL1* transgene expressed in both T and B cells, which develop mature BCL6-positive B-cell lymphomas with mutated *Ig* genes, indicating a likely origin from GC B cells.[12]

B-cell lymphomas in *pEμ-B29-TCL1* transgenic (TCL1-tg) mice develop after long latencies. This indicates that high expression of TCL1 is insufficient to drive transformation and that other genetic or epigenetic changes, presumably occurring in the GC, are required. Aberrant DNA break repair associated with the normal GC processes of *Ig* somatic hypermutation (SHM) and class switch recombination (CSR) serve as possible sources for complementing mutations to facilitate complete transformation. While cooperation of *MYC* with various companion genes, such as *BCL2*, promotes B-cell malignancy, it is unclear which genes partner with *TCL1* to facilitate B lymphomagenesis. In mature human T-cell lymphomas that have frequent *TCL1* rearrangements, abnormalities of Chr 8, which contains *MYC* at 8q24, are observed in 70% to 80% of cases, but the nature of the specific complementing genes required for transformation has not been defined.[20,21] For some human B-cell lymphomas with elevated TCL1 levels, such as BL, heightened expression of MYC caused by specific activating translocations is frequently observed in altered gene expression profiles.[22]

In the present study, we have used this TCL1-tg lymphoma model to examine how TCL1 alters GC-related functions that may contribute to B-cell transformation and to determine whether genome instability and/or altered MYC expression may complement TCL1 dysregulation in mature B-cell lymphomas.

Materials and methods

Mice

Strain-matched wild-type (WT), TCL1-tg, Oct coactivator from B cell (OCA-B)–deficient (OCA-B$^{-/-}$), and TxO (TCL1$^{+/-}$, OCA-B$^{-/-}$) mice were housed and mated in a specific pathogen–free facility as previously described.[12,23] Cells used were from 6- to 9-week-old age- and sex-matched mice with no evidence of lymphoproliferative disorder or lymphoma, in compliance with approved animal use guidelines.

Lymphocyte isolation

B and T cells were isolated by magnetic sorting using an AUTOMACS cell sorter (Miltenyi Biotech, Auburn, CA) and negative selection with anti-CD4–PE, anti-CD8–PE, anti-B220–PE, and anti-Gr1–PE antibodies (Abs) (BD Pharmingen, San Diego, CA) and anti-PE microbeads (Miltenyi Biotech), as appropriate and according to the manufacturer's instructions.

Proliferation analyses

Isolated spleen cells were cultured at 1×10^5 cells per well in 96-well plates in RPMI medium 1640 with 10% fetal calf serum (FCS) with the indicated amounts of anti–immunoglobulin M (IgM), concanavalin A, or plate-bound anti-CD3ε plus 1 μg/mL anti-CD28 monoclonal Ab (mAb). At 48 hours, cells were pulsed with 0.037 MBq (1 μCi) of [^{3}H]-thymidine for 12 hours, harvested onto glass fiber filters, and the counts per minute (cpm) were determined.

FASL-induced apoptosis

Splenic B cells (1×10^6, > 95% pure) were cultured with anti-CD40 Ab (1.5 μg/mL; BD Pharmingen) for 48 hours with or without addition of anti-IgM Ab (10 μg/mL; Jackson ImmunoResearch Laboratories, Bar Harbor, ME) during the last 24 hours. After treatment with FAS ligand (FASL, 1:25; Upstate Biotechnology, Lake Placid, NY) for 4 hours, cells were harvested and stained with Annexin V–FITC and propidium iodide (BD Pharmingen) followed by flow cytometry. FASL-induced death was determined as: % death = 100 × (% viable untreated cells − % viable cells with FASL) ÷ % viable untreated cells.

RT-PCR

Total RNA was isolated using Trizol reagent (GIBCO-BRL, Gaithersburg, MD). First-strand cDNA was synthesized from 1 μg of RNA with SuperScriptII reverse transcriptase (GIBCO-BRL). Reverse transcriptase–polymerase chain reaction (RT-PCR) primers for activation-induced cytidine deaminase *(Aicda)* and *Gapdh* are previously described.[24] A PCR protocol for *Aicda* amplification of 30, 35, and 40 cycles consisting of 94°C for 2 minutes and 59°C for 30 seconds was followed by an elongation step at 72°C for 1 minute.

Western analysis

Protein was extracted from cell pellets using NP-40 lysis buffer containing protease inhibitors, and 20 μg per sample was fractionated by SDS-PAGE followed by immunoblotting, as previously described.[17] Rabbit anti–mouse MYC antiserum (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti–mouse β-actin antiserum (1:5000; Sigma-Aldrich, St Louis, MO), or rabbit anti-TCL1 antiserum (1:15 000) were used to detect immunoreactive bands with electrochemiluminescence (ECL) reagent (Amersham Biosciences, Piscataway, NJ). Densitometry was performed using SynGene Analysis software (SynGene, Upland, CA).

Histology and tumor classification

Fixed tissues were thin sliced and stained with hematoxylin and eosin. Tumors were classified using morphologic, flow cytometric, immunohistochemical, and clonality data in accordance with Mouse Models of Human Cancer Consortium (MMHCC) criteria.[25]

Flow cytometry

Cell suspensions were depleted of red blood cells (RBCs), blocked with anti-FcγII/III receptor mAb, and surface-stained with PE- or FITC-conjugated Abs. Data were collected on a FacsCalibur (BD Pharmingen). Data were analyzed using FCS EXPRESS (De Novo Software, Thornhill, ON, Canada). Primary Abs used include anti-B220–PE, anti-IgMa–FITC, anti-IgMb–FITC, anti-CD4–PE, anti-CD5–PE, anti-CD8–FITC, and anti-Fas–PE (all from BD Pharmingen). Peanut agglutinin (PNA)–biotin (Vector Laboratories, Burlingame, CA) and antibiotin-streptavidin secondary Ab (Southern Biotech, Atlanta, GA) were also used.

Results

Enhanced proliferation by TCL1 in GC and non-GC B cells

The proliferative advantage conferred by TCL1 to peripheral lymphocytes responding to various B- and T-cell stimuli[8,12,13] may increase the opportunity for mature lymphocyte transformation events. In contrast, this growth advantage is not observed for TCL1-expressing thymocytes and immature T-cell transformation is not linked with aberrant TCL1 expression.[13] Since TCL1 mediates the transformation of GC B cells, it is possible that TCL1 has distinct proliferative effects on GC and non-GC B-cell populations. To investigate whether increased proliferation of TCL1-tg B cells occurs in both B-cell subsets in response to

stimulation through the B-cell antigen receptor (BCR), purified splenic B cells were labeled with CFSE, stimulated with anti-IgM plus interleukin-4 (IL-4) for 72 hours and then stained with Ab to B220 and PNA. IgM stimulation was chosen since almost all GC tumors that form in TCL1-tg mice express surface IgM.[12] IgM[+] GC and non-GC B cells respond to this stimulation, with B220[+]PNA[+] staining of activated B cells in vitro capturing both IgM[+] GC B cells and those that acquire a GC B-cell immunophenotype.[26] Higher percentages of both GC (B220[+]PNA[+]) and non-GC (B220[+]PNA^{−}) B cells from TCL1-tg mice had undergone multiple rounds of division compared with WT controls (Figure S1, available on the *Blood* website; see the Supplemental Material link at the top of the online article). More than 35% of TCL1-tg non-GC B cells showed more than 2 cell divisions compared with 25% for WT non-GC B cells (ratio, approximately 1.4:1). Similarly, more than 40% of TCL1-tg GC B cells showed more than 2 cell divisions compared with less than 25% for WT GC B cells (ratio, approximately 1.6:1). These findings suggested that TCL1 does not provide a GC B-cell–specific proliferative advantage and that other functions related to the GC also contribute to the transformation of GC B cells.

B cells with heightened TCL1 expression are more resistant to CD40-mediated, FASL-induced apoptosis

Transformed B cells from TCL1-tg mice exhibit features of GC rather than pre-GC or post-GC B cells. This occurrence cannot be explained by the enhanced proliferation of nontransformed TCL1-tg B cells, regardless of their tendency to adopt a GC or non-GC fate. This prompted us to assess the effects of heightened TCL1 expression on a prominent feature of the GC reaction, cell death induced by the FAS/FASL signaling pathway. Naive, mature primary mouse B cells express little or no FAS.[27] CD40-expressing B cells recruited into the GC engage CD40L on CD4^{+} T cells, which induces B-cell activation and the surface expression of FAS.[28] CD40L-engaged GC B cells then become susceptible to FASL-induced cell death. Similarly, stimulation of naive B cells with CD40L (CD154) in vitro induces FAS expression on B cells and FASL sensitivity to cell death.[28] Subsequent engagement of the BCR by antigen (Ag) protects CD40-activated B cells from FAS-induced death by increasing the expression of BCL2L1 (Bcl-X_L) and CFLAR (Flip).[29-31] This BCR-mediated rescue signal probably acts to reinforce appropriate B-cell–T-cell interactions and eliminate illegitimate, non-Ag–driven B-cell expansion in the GC.[32,33] For this study, purified B cells from TCL1-tg and WT mice were activated with anti-CD40 and then treated with FASL in the presence or absence of anti-IgM Ab. Cells from TCL1-tg mice treated with anti-CD40 and FASL in the absence of BCR ligation had less than half the sensitivity of WT B cells to induction of apoptosis (Figure 1A). This disparity could not be attributed to differences in expression of surface FAS induced by anti-CD40, as comparable levels were detected on CD40-activated cells of either genotype (Figure 1B). Interestingly, B cells from mice of both genotypes were rescued to comparable extents by BCR ligation. These results demonstrated that enhanced expression of TCL1 induced increased resistance to FAS-mediated death in the absence of BCR engagement and rescue signaling. This survival advantage conferred by TCL1 could result in the persistence of GC B cells otherwise destined to die, and these cells might be the targets of mutagenic events that result in transformation.
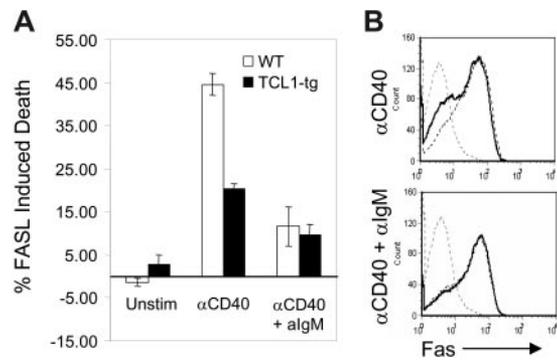

Figure 1. TCL1 protects CD40-stimulated B cells from FASL-induced apoptosis. (A) Isolated B220[+] spleen B cells from WT (□) or TCL1-tg (■) mice were treated with 1.5 μg/mL anti-CD40 followed by incubation with FASL (1:50) with or without 10 μg/mL anti-IgM. The percentage of death induced with each condition was determined as described in "Materials and methods." (B) Surface FAS expression was evaluated in purified B cells isolated from WT and TCL1-tg mice. Cells were either unstimulated (gray hatched line, both WT and TCL1-tg) or treated with anti-CD40 (1.5 μg/mL) with or without 10 μg/mL anti-IgM (WT, hatched line; TCL1-tg, solid black line). The data are representative of 3 separate experiments, and error bars represent standard deviation between experiments.

Aicda expression is augmented in stimulated TCL1-tg B cells

Expression of *AICDA* has been reported for GC B cells,[24,34,35] and among human NHL expression is mainly confined to those of GC phenotype.[35-38] Human DLBCL exhibit a series of genetic alterations that can result from inappropriate repair of DNA double-strand breaks caused by AICDA during CSR and SHM. These changes include Chr translocations and activating mutations in the 5′ regulatory regions of proto-oncogenes such as *BCL6*, *MYC*, and *PIM1*.[39] In addition, mice overexpressing *Aicda* from a transgene develop T-cell lymphomas with high levels of mutations in *TCR* and *MYC* genes, with no recurring gross Chr anomalies, and increased CSR in stimulated GC B cells.[40,41]

TCL1 enhances B220[+]PNA[+] GC B-cell proliferation (Figure S1) and increased *Aicda* expression has recently been linked to an increased rate of cell division.[41] To determine whether *Aicda* expression is elevated in TCL1-tg B cells as a possible contributor to lymphoma development, we examined *Aicda* expression in purified B cells of TCL1-tg and WT mice using semiquantitative RT-PCR. Transcripts were not detected in rested and unstimulated cells from mice of either genotype but were induced in cells coactivated with lipopolysaccharide (LPS) plus IL-4 or anti-CD40 plus IL-4 (Figure 2).[42] When normalized to expression of *Gapdh*, *Aicda* transcripts were approximately 2- to 3-fold higher in TCL1-tg than WT B cells activated by either approach. These results demonstrated that heightened *Aicda* expression in TCL1-tg B cells from exogenous stimulation could have a role in GC B-cell transformation by a predicted increase in genome instability.[43,44]

A GC microenvironment is required for TCL1-tg mice to develop lymphomas

The findings thus far indicate that several effects of TCL1 on B cells could set the stage for development of B-cell lymphomas of GC phenotype: increased responsiveness to proliferative stimuli for both B220^{+}PNA^{+} GC B cells (Figure S1) and supporting CD4^{+} T cells;[13] decreased sensitivity to CD40-mediated FASL-induced apoptosis (Figure 1); and increased expression of *Aicda* with enhanced cell proliferation (Figure 2). While we hypothesize that the GC is required for TCL1-mediated mature B-cell transformation, the fact that TCL1-tg lymphomas express BCL6 and exhibit SHM of *Ig* genes suggests, but does not prove, that they require the
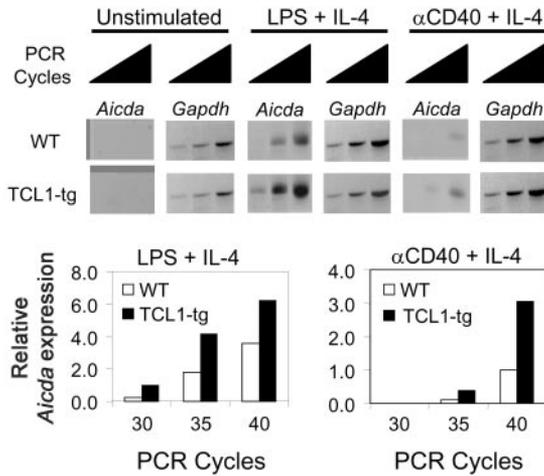
Figure 2. TCL1 enhances *Aicda* expression in B cells. Isolated B220+ spleen B cells from WT or TCL1-tg mice were treated with LPS (10 μg/mL) plus IL-4 (50 U/mL) or anti-CD40 (1.5 μg/mL) plus IL-4 (50 U/mL), followed by 30, 35, and 40 cycles of RT-PCR to detect relative *Aidca* and *Gapdh* mRNA levels. The data are representative of 2 repeat experiments.
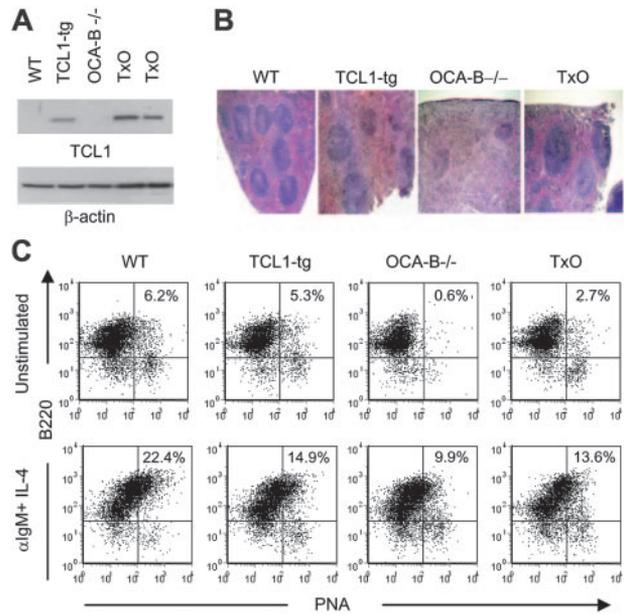

Figure 3. Characterization of 6- to 8-week-old TxO mice. (A) TCL1 expression is shown by Western blot for WT, TCL1-tg, OCA-B$^{-/-}$, and TxO spleen cells. β-actin expression is shown in the bottom panel. (B) H&E staining of WT, TCL1-tg, OCA-B$^{-/-}$, and TxO spleens. Original magnification, ×40. Visualization was performed using an Olympus BX40 microscope with a UPlan 4×/0.13 NA air objective (Olympus America, Melville, NY). Images were collected with an Olympus DP10 camera and processed with Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). (C) Detection of B220+PNA^{hi} B cells by flow cytometry. Cells were untreated (top panels) or were stimulated with anti-IgM plus IL-4 (bottom panels).

GC environment to develop. To test this important hypothesis, TCL1-tg mice were crossed with mice deficient in the transcriptional coactivator OCA-B (OCA-B$^{-/-}$), to generate mice (TxO) incapable of forming GC. Previous studies have shown varying effects of OCA-B deficiency on the development of bone marrow, B1, B2, and marginal zone B cells, with at least some differences being strain dependent. However, a lack of GC development with an intact B-cell response to T-independent antigen stimulation and terminal differentiation to antibody-secreting cells were constant findings.[45,46] Interestingly, the generation of T-dependent antibody-secreting cells was blocked and caused the accumulation of GC-phenotype founder B cells that express BCL6 and Aicda and which can undergo SHM and CSR.[46] These T-dependent OCA-B$^{-/-}$ GC-like B cells resemble the B cells that form GC B-cell lymphomas in TCL1-tg mice.[12]

To determine whether a deficiency in OCA-B would affect TCL1-mediated lymphocyte responses in the TCL1-tg mice, we first studied TCL1 protein levels in spleens of TCL1-tg and TxO mice. OCA-B could affect expression of the *TCL1* transgene that is regulated by OCA-B–sensitive *B29* promoter and *Eμ* enhancer elements.[47] However, Western blot analyses showed that the levels of TCL1 protein expression were similar in the spleens of TxO and TCL1-tg mice (Figure 3A) and less than seen in T-cell leukemias with *TCL1* gene rearrangements.[13] TxO bone marrow did not show the developmental block that characterizes early B-cell differentiation in OCA-B$^{-/-}$ mice, and TxO marrow had similar to slightly higher frequencies of immature, transitional, and recirculating B cells than did OCA-B$^{-/-}$ bone marrow (Table S1).[48]

Histologically, the spleens of TxO mice, like those of OCA-B$^{-/-}$ mice, were devoid of GCs (Figure 3B). TxO spleens differed from those of OCA-B–deficient mice in having much larger primary follicles. This effect could be ascribed to expression of the *TCL1* transgene, since the primary follicles of TCL1-tg mice were considerably larger than those of WT animals (Figure 3B). Consistent with a lack of GCs, the frequencies of B cells with a B220+PNA+ GC phenotype in spleens from OCA-B$^{-/-}$ mice were markedly reduced when compared with WT spleens (Figure 3C). In TxO spleens, the frequencies of B220+PNA+ cells were increased more than 4-fold from the levels in OCA-B$^{-/-}$ spleens. This suggests that heightened expression of TCL1 might promote the expansion of cells with the B220+PNA+ GC phenotype[49] that are

primed to enter nascent GCs, even in mice incapable of GC formation. Further comparisons of TxO and OCA-B$^{-/-}$ mice showed that TCL1 increased the proliferation responses of splenocytes to BCR ligation, LPS, concanavalin A, or anti-CD3 plus anti-CD28 (Figure 4). These results indicated that the *TCL1* transgene was well expressed in the OCA-B–deficient background, and that the follicular B cells of OCA-B–deficient mice, while responsive to the proliferative effects of TCL1, were still unable to form GCs.
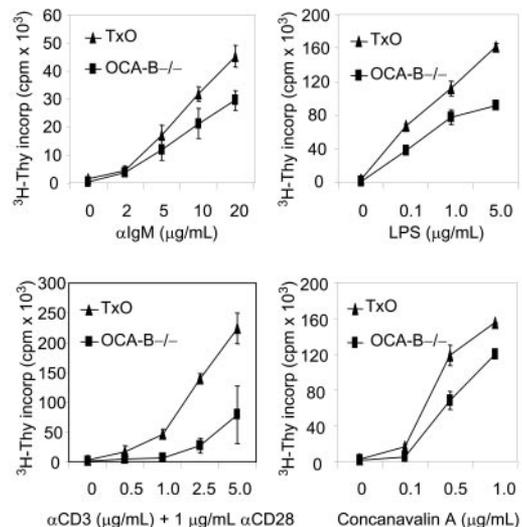

Figure 4. TCL1 enhanced growth of stimulated OCA-B$^{-/-}$ non-GC B and T cells. Isolated OCA-B$^{-/-}$ and TxO splenocytes were incubated with the listed factors and assayed at 48 hours for tritiated thymidine incorporation. Error bars represent standard deviation in triplicate measurements at each assay point.
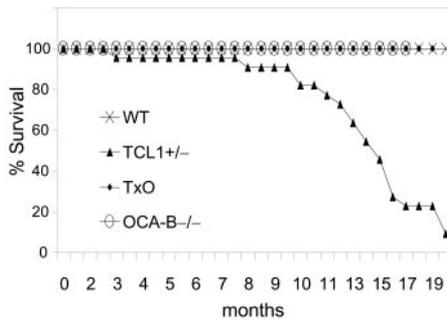
Figure 5. The GC is required for B-cell lymphomagenesis in *pEμ-B29-TCL1* transgenic mice. Kaplan-Meier survival plot is shown. WT (n = 20), TCL1-tg (n = 22), OCA-B$^{-/-}$ (n = 23), and TxO (n = 25) mice were monitored up to 20 months or until they became moribund, followed by killing and necropsy.

We then compared TCL1-tg, TxO, OCA-B$^{-/-}$, and WT mice for tumor development (Figure 5). TCL1-tg mice first became ill at around 3 to 4 months of age, and by 20 months, 86% of the mice (19 of 22) had been killed when moribund with advanced disease. In contrast, none of the WT, OCA-B$^{-/-}$, or TxO mice became ill during this time period. At necropsy, TCL1-tg mice had greatly enlarged spleens that most often contained monomorphic populations of IgM$^+$B220$^+$CD5^{dull} B cells (Figure S2; Table S2) that were clonal for *IgH* gene rearrangements by Southern blot analysis (Figure S3). Lymphadenopathy was common due to diffuse subcortical accumulations of the same cells found in the spleen, often with accompanying histiocytosis. Hepatomegaly was also frequent with perivascular infiltrates of malignant lymphoid cells and histiocytes. Some cases exhibited lymphomatous infiltrates of the lung, kidney, or salivary gland. By histopathologic criteria, the lymphomas that developed were all of GC or post-GC origin, being comprised of FL, DLBCL, BLL, and plasmablastic lymphomas. Two cases, 1 plasmablastic and 1 BLL, had associated leukemic phases, and many were associated with prominent accumulations of TCL1-negative histiocytes (Figure S4). Most lymphomas exhibited intense nuclear staining for PAX5 (Figure S2) and BCL6, as shown previously.[12] Overall, these results indicate that B cells of TCL1-tg mice were critically dependent on exposure to the GC environment for neoplastic transformation.

TCL1-tg BLLs are marked by chromosomal alterations and enhanced MYC expression

Recurring genetic alterations in human B-cell lymphomas include chromosomal translocations as well as point mutations in regulatory sequences of *MYC*, *PIM1*, and *BCL6* genes that can result in

cell activation. The long latency for lymphoma development in TCL1-tg mice indicated that *TCL1* dysregulation was insufficient for transformation, and that other genetic alterations were required for the appearance of overt lymphomas. To determine whether gross chromosome changes might be involved, we used spectral karyotype (SKY) analysis to examine metaphase spreads from 5 primary lymphomas comprising 4 BLLs and 1 histiocyte-associated DLBCL (Table 1; Figure S5). All BLLs had reciprocal Chr translocations, including 3 involving Chr 5: 2 T(2;5) and 1 T(5;12). In addition, all BLLs were trisomic for Chr 15 (trisomy 15 [Ts(15)]). The single DLBCL tested by SKY had neither translocations nor Ts(15).

The fact that *Myc* is located on Chr 15, and that Ts[15] found in mouse thymic T-cell lymphomas are associated with overexpression of *Myc*,[50,51] suggested that TCL1-associated BLL might also express MYC at increased levels. We examined this possibility by performing western analyses of MYC levels in extracts from 6 BLLs and 3 DLBCLs. The results (Figure 6; Table 1) revealed heightened expression of MYC in cases with and without Ts(15). MYC expression is aberrant and not passively increased from TCL1-enhanced proliferation since IgM$^+$ centroblasts, from which TCL1-tg IgM$^+$ BLLs probably arise, are the most rapidly proliferating cells in humans, and yet they do not express MYC.[52] Further supporting this notion, nonstimulated TCL1-tg B cells do not have a proliferative advantage over WT B cells in culture (Figure S1), and the level of MYC expression in in vitro–activated TCL1-tg B cells is unchanged compared with WT B cells (data not shown).

Mutations found in the 5′ regulatory sequences of the *MYC* proto-oncogene in human GC lymphomas have features strongly suggesting that they are caused by AICDA.[39] In support of this notion, *Aicda* transgenic mice develop T-cell lymphomas with heavily mutated sequences 5′ of the *Myc* gene.[40] Since enhanced *Aicda* expression is associated with TCL1 expression (Figure 2), it seemed possible that heightened expression of MYC in the TCL1-tg lymphomas could be due to mutations 5′ to the gene. To test this possibility, we sequenced the 5′ regions in 2 lymphomas that exhibited SHM of *IgH*, 1 with and 1 without Ts(15) (Table S3). In neither tumor was the frequency of mutations increased over that found in WT spleen cells, despite the fact that most TCL1-tg tumors exhibit moderate to high-levels of *Aicda* expression (Figure S6).

Discussion

The findings presented here provide new insights into the mechanisms by which TCL1 dysregulation sets the stage for B-cell

Table 1. SKY and MYC expression of TCL1-tg B-cell lymphomas

Tumor	MMHCC	Translocation	No. metaphases (no. abnormal metaphases)	Trisomy 15	MYC
K1	DLBCL	ND	ND	ND	0.7
K2	BLL	ND	ND	ND	1.2
K7	BLL	T(11;17)	16 (16)	+	3.2
K9	DLBCL	None	11 (0)	−	2.2
K10	BLL	T(2;5),T(X;7),T(7;9),T(9;X)	9 (8)	+	1.8
K14	DLBCL	ND	ND	ND	1.0
K15	BLL	ND	ND	ND	1.8
K18	BLL	T(5;12)	14 (12)	+	2.0
P3	BLL	der(5),T(2;5)	8 (8)	+	3.1
WT	NA	ND	ND	ND	~0

NA indicates not applicable; ND, not done.
Tumors were classified using MMHCC criteria.[12,25] All translocations were reciprocal. MYC protein expression levels are listed as ratios to sample K14, set at 1.0.
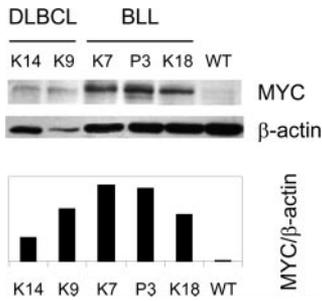
Figure 6. Elevated expression of MYC protein in TCL1-tg DLBCL and BLL. A Western blot for MYC and β-actin expression is shown for BLL (K7, P3, and K18), DLBCL (K9, K14) and control (WT) spleen cells.[12] The ratio of MYC to β-actin expression was determined by densitometry.

transformation, the cellular interactions required for evolution of the transformed phenotype, and the role of genomic instability in the emergence of malignant lymphomas (Figure 7).

TCL1 is normally expressed in bone marrow B-cell progenitors, migrants to secondary lymphoid compartments, and mature cells in the mantle zone, but is down-regulated during the GC reaction and is silenced in post-GC memory B and plasma cells.[17] A role for TCL1 in differentiation is not known, but its physiologic function is likely tied to its ability to enhance the signaling activity of AKT and PKC-MAPK-ERK pathways,[8,9,13] thereby affecting both cell survival and proliferation. Direct evidence for this came from studies here of isolated TCL1-tg B cells that demonstrated enhanced sensitivity to GC-related proliferative signals and increased resistance to CD40L-dependent, FAS-mediated apoptosis. Inhibition of CD40-dependent cell-death pathways may contribute importantly to GC B-cell transformation as widespread FAS-dependent apoptosis is a striking feature of GC dark zone centroblasts.[52]

Enhanced activation of AKT is unlikely to be sufficient for transformation, however, as mice with B cells deficient in PTEN, a negative regulator of AKT, did not develop B-cell lymphomas, and a constitutively active AKT caused cell senescence.[54,55] Constitutive activation of AKT in mice with PTEN-deficient B cells also results in decreased and not increased expression of Aicda and reduced CSR.[54] Aicda repression from senescence-inducing PTEN-deficiency contrasts sharply with enhanced *Aicda* expression from proliferation-inducing TCL1 expression shown here. This key distinction links TCL1 but not PTEN deficiency to cell-cycle–dependent *Aicda* expression[41] and, more importantly, provides a mechanistic explanation for why TCL1 expression but not PTEN deficiency promotes mature B-cell transformation by increasing rather than decreasing genome instability.[43,44] Genome alterations are usually opposed by a p53-dependent checkpoint, which protects the genome by forcing damaged cells into cell-cycle arrest or apoptosis. However, GC B cells present a special situation in which p53 is normally repressed by BCL6, possibly to permit Aicda-dependent SHM and CSR without deleting antigen-selected B cells.[53] TCL1 expression might further reinforce p53 silencing in this transgenic model by activating AKT and MAPK signaling pathways, which phosphorylate Mdm2 and reduce p53 expression.[56] By a similar AKT-dependent mechanism, PTEN deficiency could also decrease p53 expression, but a simultaneous reduction in Aicda would be predicted to protect GC B cells from enhanced DNA damage and impair tumor production.[54,57] These findings suggest that additional AKT-dependent or alternative AKT-independent mechanisms downstream of TCL1 contribute to malignant transformation, perhaps through activation of the PKC-MAPK-ERK signaling pathway.[13] Supporting this notion, a recent

study has shown a role for MAPK activation in controlling Aicda expression in mouse B cells.[58]

In addition to providing a critical distinction between TCL1 expression and PTEN deficiency that could control GC B-cell transformation, a striking conclusion from our studies was that development of lymphomas in TCL1-tg mice was absolutely dependent on expression of the transcriptional coactivator OCA-B. Although a deficiency in OCA-B has been shown to affect several aspects of B-cell development in a somewhat strain-dependent manner, an OCA-B knockout on any strain background is incapable of generating GCs.[45] We showed that the effect on lymphoma development could not be ascribed to major changes in early B-cell development, altered frequencies of follicular B cells, or inhibition of TCL1 transgene expression in OCA-B$^{-/-}$ B cells. From this, we infer that signals inherent to the GC microenvironment are critical to the transformation of B cells with aberrant regulation of TCL1, and that Aicda expression is necessary[43,44] but not sufficient, since activated OCA-B$^{-/-}$ B cells[46] express Aicda and do not form cancers. One source of these signals in our model could be T cells that also express the *TCL1* transgene. This possibility is suggested by the observation that FL appearing in mice bearing a *BCL2* transgene expressed in both B and T cells fail to develop if the mice are depleted of CD4$^+$ T cells, which could be required to sustain GC expansion until additional B-cell–transforming events emerge.[59] It should be noted that marginal zone B cells are variably reduced in number and function in OCA-B–deficient mice, and that marginal zone B-cell lymphomas (MZLs) with features of centroblasts have been described.[45,60] MZLs with features of BLL, a common class of TCL1-tg lymphoma, have not been described, making it less likely that the OCA-B$^{-/-}$ effect on lymphoma development can be ascribed to changes in the marginal zone. It is noteworthy that histiocytes feature prominently in many TCL1-tg
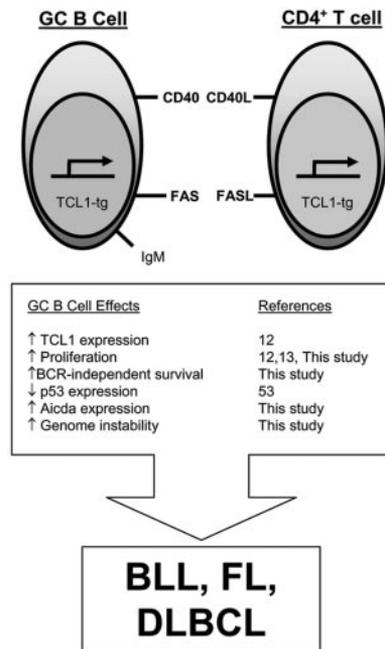

Figure 7. Model for TCL1-tg effects on GC B cells that promote malignant transformation. Aberrantly elevated *pEμ-B29-TCL1* expression in the GC enhances lymphocyte proliferation, increases resistance to FAS-induced cell death, and augments *Aicda* expression. Since GC B cells have reduced p53 expression,[53] these tumorigenic features occur in a setting that is relatively permissive for usual (SHM, CSR) and abnormal genomic alterations, increasing the likelihood for GC B-cell lymphoma development.

lymphomas, and recent studies have identified the tumor microenvironment and host inflammatory responses mediated by products of macrophages and dendritic cells as defining features in a subset of human DLBCL.[61] Since histiocytes do not express the *TCL1* transgene (Figure S4), their association with TCL1-tg lymphomas suggests they are recruited to the tumors in response to signals originating from TCL1-expressing B or T cells.

Analyses of TCL1-tg lymphomas by SKY provided additional insights into the mechanisms that complement TCL1 dysregulation for transformation. Recurrent Ts(15) associated with heightened expression of MYC was perhaps most revealing and provided the first reported alterations in tumors induced by dysregulated *TCL1* family gene expression in transgenic mice with strong parallels to human B-cell malignancies. Dysregulated expression of MYC secondary to Ig-MYC translocations is the hallmark feature of human BL and BLL. Additionally, dysregulated MYC has been shown to impair double-stranded DNA break repair.[62,63] In some cell types, these changes are insufficient to induce transformation, indicating a need for complementing mutations. Studies of *MYC* transgenic mice using proviral insertional mutagenesis to tag interacting oncogenes identified several complementation groups for transformation. These include *Pal/Bmi1/Gfi1* in 1 set, members of the *Pim* family of serine threonine kinases in a second, and *Runx2* in a third.[64] Although *Tcl1* was not identified in these screens, it is possible that *MYC* and *TCL1* form yet another set of complementing genes. This suggestion gains weight from the observation that MYC is overexpressed in human T-cell lymphomas bearing *TCL1* translocations[20,65] and could be tested by crossing transgenics in which both *MYC* and *TCL1* are targeted to the B-cell lineage.

SKY analyses of the TCL1-tg lymphomas also revealed recurring translocations involving Chr 5 and others involving Chr 2, 7, 9, 11, 17, and X. Although the significance of these events is currently not known, the T(5;12) may provide an opportunity to clone the gene at the Chr 5 breakpoint if the *IgH* locus on Chr 12 is involved

in the translocation. The fact that stimulated TCL1-tg B cells are protected from death and express increased levels of *Aicda* may be relevant to the frequency with which translocations were observed in these lymphomas. In the plasmacytomas of IL-6 transgenic mice or in retrovirally transduced primary B cells, for example, it is clear that *Aicda* is absolutely required but may not be sufficient for the development of *Igh/Myc* translocations.[43,66] Furthermore, in a pristane-induced model of lymphomagenesis, *Aicda* was found to be required for the outgrowth of transformed cells.[44] Therefore, increased levels of *Aicda* in our model suggest a role for TCL1 in supporting the development of genetic lesions and maintaining the cells that harbor them.

Taken together, these results establish that *pEμ-B29-TCL1* lymphomas require the GC to develop and strongly suggest that transformation arises as a result of tumorigenic changes to key GC-related functions and pathways. Our data further point to TCL1 effects on BCR signaling and CD40L-dependent FAS-induced death pathways that set the stage for GC-associated increases in Aicda expression in p53-suppressed B cells, leading to tolerated genetic errors including aneuploidy and aberrant MYC activation.

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