TCL1 Oncogene Expression in B Cell Subsets from Lymphoid Hyperplasia and Distinct Classes of B Cell Lymphoma

Jonathan W. Said, Katrina K. Hoyer, Samuel W. French, Lisa Rosenfelt, Maria Garcia-Lloret, Patricia J. Koh, Tse-Chang Cheng, Girija G. Sulur, Geraldine S. Pinkus, W. Michael Kuehl, David J. Rawlings, Randolph Wall, and Michael A. Teitell

Department of Pathology and Laboratory Medicine (JWS, KKH, SWF, LR, TCC, GGS, MAT), Department of Pediatrics (MG-L, DJR), and Jonsson Comprehensive Cancer Center (DJR, RW, MAT), University of California at Los Angeles School of Medicine, and Department of Microbiology and Immunology (PJK, RW) and Molecular Biology Institute (DJR, RW, MAT), University of California at Los Angeles, Los Angeles, California; Department of Pathology (GSP), Brigham and Woman's Hospital, Harvard Medical School, Boston, Massachusetts; and Genetics Department (WMK), Medicine Branch, National Cancer Institute, Bethesda, Maryland

SUMMARY: Activation of the *TCL1* oncogene has been implicated in T cell leukemias/lymphomas and recently was associated with AIDS diffuse large B cell lymphomas (AIDS-DLBCL). Also, in nonmalignant lymphoid tissues, antibody staining has shown that mantle zone B cells expressed abundant Tcl1 protein, whereas germinal center (GC; centrocytes and centroblasts) B cells showed markedly reduced expression. Here, we analyze isolated B cell subsets from hyperplastic tonsil to determine a more precise pattern of Tcl1 expression with development. We also examine multiple B cell lines and B lymphoma patient samples to determine whether different tumor classes retain or alter the developmental pattern of expression. We show that *TCL1* expression is not affected by Epstein-Barr virus (EBV) infection and is high in naïve B cells, reduced in GC B cells, and absent in memory B cells and plasma cells. Human herpesvirus-8 infected primary effusion lymphomas (PEL) and multiple myelomas are uniformly *TCL1* negative, whereas all other transformed B cell lines tested express moderate to abundant *TCL1*. This observation supports the hypothesis that PEL, like myeloma, usually arise from post-GC stages of B cell development. Tcl1 protein is also detected in most naïve/GC-derived B lymphoma patient samples (23 of 27 [85%] positive), whereas most post-GC–derived B lymphomas lack expression (10 of 41 [24%] positive). These data indicate that the pattern of Tcl1 expression is distinct between naïve/GC and post-GC–derived B lymphomas ($P < 0.001$) and that the developmental pattern of expression is largely retained. However, post-GC–derived AIDS-DLBCL express *TCL1* at a frequency equivalent to naïve/GC-derived B lymphomas in immune-competent individuals (7 of 9 [78%] positive), suggesting that *TCL1* down-regulation is adversely affected by severe immune system dysfunction. These findings demonstrate that *TCL1* expression in B cell lymphoma usually reflects the stage of B cell development from which they derive, except in AIDS-related lymphomas. (*Lab Invest 2001, 81:555–564*).

T cell leukemias/lymphomas in adults and in children with the genomic instability syndrome ataxia telangiectasia have repeated rearrangements on chromosome 14q32.1 (Davey et al, 1988; Mengle-Gaw et al, 1988; Russo et al, 1988; Virgilio et al, 1993). Analysis of a 160-kb region on 14q32.1 that is bracketed by two breakpoint clusters led to the identification of a candidate tumor-promoting gene, *TCL1* (Virgilio et al, 1994).

14q32.1 chromosomal rearrangements reposition T cell receptor control elements at 7q35 or 14q11 near the *TCL1* coding region, resulting in abnormal gene activation in T cell malignancies. Recently, the Tcl1 oncoprotein was found by coimmunoprecipitation and yeast two-hybrid studies to interact with the essential serine/threonine kinase Akt (Laine et al, 2000; Pekarsky et al, 2000). These studies indicated that Tcl1 activates Akt, resulting in increased phosphorylation of Akt-target proteins, enhanced cell proliferation and survival, and Akt nuclear relocalization. These findings provide a mechanism for Tcl1-dependent T cell transformation through inappropriate Akt pathway activation. It is unknown if aberrant Tcl1 expression also induces B cell malignancies as well.

In an initial survey of B cells, *TCL1* expression was shown primarily in bone marrow and in endemic, Epstein-Barr virus (EBV)-positive Burkitt lymphoma

(BL) cell lines (Virgilio et al, 1994). However, a subsequent study yielded data that also showed *TCL1* expression by mature peripheral B cells (PBC) (Takizawa et al, 1998). With regard to EBV infection, this second study did not assess the EBV status of PBC, and more than 90% of individuals worldwide are known to harbor latent EBV (Westphal et al, 1999). Complicating this picture was a report that immortalization of PBC with EBV yielded clones that were uniformly *TCL1*-positive (Pekarsky et al, 1999). The combined data suggested that EBV infection might positively influence *TCL1* expression in PBC and BL.

We recently investigated normal, nonmalignant *TCL1* expression in reactive lymphoid tissues by immunohistochemistry (Teitell et al, 1999). We found that nonproliferating mantle zone B cells expressed very high levels of Tcl1 protein, whereas germinal center (GC) B cells showed markedly reduced levels of expression. In addition, a large majority of interfollicular zone lymphocytes failed to express Tcl1. This specific pattern of Tcl1 expression suggested that B cell lymphomas derived from distinct follicular zones would express characteristic levels of Tcl1, whereas post-GC B cell lymphomas would likely not express Tcl1. However, we also identified Tcl1 expression in an EBV-negative, AIDS diffuse large B cell lymphoma (AIDS-DLBCL) by subtractive gene hybridization (Teitell et al, 1999). An extended survey of patient tumor tissues showed that a majority of AIDS-DLBCL expressed *TCL1* (6 of 8 [75%] positive). AIDS-DLBCL arise from post-GC B cells and our observation suggested that *TCL1* may be expressed aberrantly and have a role in tumor development. Because EBV status did not correlate with Tcl1 expression in AIDS-DLBCL, it is possible that the functional status of the immune system has a role in *TCL1* regulation and that B cell transformation is augmented by or affects Tcl1 expression.

Here, we investigate *TCL1* expression in normal B cells, nontransformed and transformed B cell lines, and in a wide spectrum of patient-derived B cell malignancies from immune-competent individuals. This study was prompted by our immunohistochemical findings that suggested that post-GC B lymphocytes exhibit down-regulation of Tcl1 expression, whereas AIDS-DLBCL appear to retain or re-express Tcl1 (Teitell et al, 1999). The main question addressed is whether Tcl1 expression is altered in distinct classes of B lymphoma and therefore could potentially contribute to malignant transformation in immune-competent and immune-deficient individuals.

Results

TCL1 Expression is Independent of EBV Infection in B Cell Lines

Northern analyses of TCL1 expression were performed on immortalized and transformed B cell tumor lines containing or lacking EBV. The data show that *TCL1* levels remain stable with or without EBV infection of endemic or sporadic BL (Fig. 1 and Table 1). In addition, lack of EBV-encoded major oncoproteins EBNA-2 and LMP1 did not abolish *TCL1* expression in the P3HR-1 BL line (Miller et al, 1974). Establishment of a mature immune system, with reactive secondary GC, is not required for *TCL1* expression in circulating PBC, inasmuch as 9 of 9 immortalized fetal cord B cell lines and 6 of 6 immortalized PBC from normal adults express abundant *TCL1* (Ounanian et al, 1992; Pekarsky et al, 1999). Furthermore, four primary effusion lymphomas (PEL) lines (and two PEL patient samples) failed to express *TCL1* regardless of EBV infection (Fig. 1, Table 1, and data not shown). Overall, the data show that a mature immune system is not required for Tcl1 expression and that EBV infection is not associ-
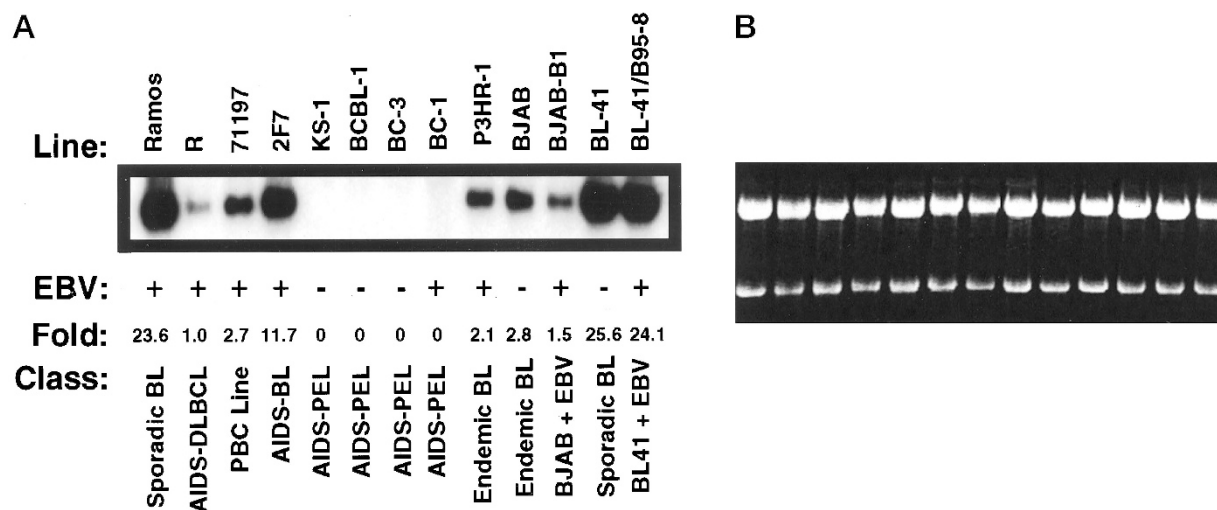

Figure 1.
TCL1 mRNA expression in B cell lines is independent of Epstein-Barr virus (EBV) status. A, Northern analysis of *TCL1* expression. Fold-induction is determined by Phosphorimager analysis, and the values listed are relative to an arbitrarily set value of 1.0 for the AIDS diffuse large B cell lymphoma (AIDS-DLBCL) cell line R. EBV status is derived from published reports. *BL*, Burkitt lymphoma; *DLBCL*, diffuse large B cell lymphoma; *PBC*, peripheral B cell; *PEL*, primary effusion lymphoma. B, Ethidium bromide stained formaldehyde gel showing equal lane loading of total RNA from B cell lines.

Table 1. Relative *TCL1* mRNA Expression Levels in Immortalized and Transformed B and T Cell Lines[a]

Category	Line Designation	Classification	HHV-8 Status	EBV Status	Ig	*TCL1* (Fold)
Immortalized	Fetal cord	n = 9	−	+	+	1.0–23.8
lines	PBC	n = 6	−	+	+	2.7–10.8
Burkitt	BL41	Sporadic	−	−	+	25.6
lymphoma	BL41/B95–8	EBV-infected	−	+	+	24.1
lines	BJAB	Endemic	−	−	+	2.8
	BJAB/B1	EBV-infected	−	+	+	1.5
	Akata A1.5	Sporadic	−	−	+	5.7
	Akata 2A8	EBV-infected	−	+	+	5.3
	Akata 3F2	EBV-infected	−	+	+	5.8
	Ramos	Sporadic	−	−	+	23.6
	P3HR-1	Endemic	−	+	+	2.1
AIDS lines	2F7	BL	−	+	+	11.7
	R	DLBCL	−	+	+	1.0
PEL lines	KS-1		+	−	−	0
	BC-1		+	+	−	0
	BC-3		+	−	−	0
	BCBL-1		+	−	−	0
Myeloma	Various	n = 29	−	ND	ND	0
T cell lines	Jurkat	T-ALL	−	−	−	0
	CEM	T-ALL	−	−	−	0
Myeloid line	K562	CML	−	−	−	0

BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; ND, not determined.

[a] Summary of cell lines examined, HHV-8 and EBV status, Ig production, and Northern analysis for *TCL1* gene expression. HHV-8, EBV, and Ig values were obtained from published reports except for fetal cord and PBC lines (Benjamin et al, 1982; Demidem et al, 1996; Miller and Lipman, 1973; Ruf et al, 1999). Nine fetal cord, 6 PBC, and 29 myeloma cell lines were surveyed for *TCL1* expression. The myeloma lines used here have been described previously (Shou et al, 2000). P3HR-1 is an endemic BL that lacks major EBV oncoproteins EBNA-2 and LMP1. The data for *TCL1* RNA expression is presented as a relative level of expression over the arbitrarily set value of 1.0 for cell line R (Fig. 1).

ated with nor does it affect Tcl1 expression in transformed B cell lines.

PEL and Myeloma Transformed Cell Lines Do Not Express Tcl1

PEL are a unique and rare category of immunodeficiency-related lymphoma that almost always contain human herpesvirus-8 and are most often associated with advanced stages of AIDS (Drexler et al, 1998; Jones et al, 1998). PEL mainly are considered a GC- or post-GC–derived B lymphoma because of their variable large cell morphology, frequent activation marker expression, and productive rearrangements of their nonexpressed immunoglobulin genes with evidence of somatic hypermutation (Drexler et al, 1998; Matolcsy et al, 1998). However, the AIDS-related PEL line BC-3 recently has been shown to lack evidence of immunoglobulin gene mutations, suggesting that this PEL may originate from a naïve/pre-GC rather than a GC or post-GC B cell precursor (Matolcsy et al, 1998). The pattern of Tcl1 expression, therefore, was assessed in four different PEL lines (and in two PEL patient samples) in an attempt to provide additional insight into the derivation of this unusual lymphoma subclass. In 6 of 6 cases, including the BC-3 PEL cell line, Tcl1 expression was not detected (Fig. 1, Table 1, and not shown). This

pattern most closely resembles an earlier report of negative Tcl1 expression in post-GC and terminally differentiated myeloma cell lines (Takizawa et al, 1998). To confirm and extend this earlier reported expression pattern into patient tumor samples, we assessed an additional 29 myeloma cell lines, along with three patient-derived myeloma tissues, and all were found to be negative for Tcl1 expression (Table 1, and not shown).

Tcl1-Expression in Normal Peripheral B Cells Depends on Developmental Stage

Previous antibody staining of lymphoid tissues suggested a developmental stage–dependent change in Tcl1 expression in B cells (Teitell et al, 1999). To clearly define Tcl1 expression levels in B cell subsets, we isolated distinct populations from reactive human tonsils by a magnetic bead cell sorting system (MACS). Naïve B cells (CD10+/IgD+) express abundant Tcl1, whereas GC-derived B cells (CD10+/IgD-) express markedly reduced Tcl1 (19% of naïve B levels), and memory B cells (CD10-/IgD-) and plasma cells (CD138+) do not express Tcl1 (Fig. 2). These findings are consistent with earlier immunohistochemistry results and indicate that scattered, interfollicular zone Tcl1+ cells are not post-GC B cells (Teitell et al,

1999). Additional support for a staged down-regulation in Tcl1 expression with B cell maturation was obtained with an in vitro coculture system in which CD19+ B cells are stimulated to differentiate into Ig-secreting plasmacytoid cells or plasmablasts (Fluckiger et al, 1998) (Fig. 2). MACS-isolated CD19+ tonsillar lymphocytes were cocultured with CD40L-expressing L cells in the presence of IL4 and IL10 and were harvested after 7 days. Wright-Giemsa staining demonstrated that these B cells had obtained plasmacytoid morphology, with eccentrically placed nuclei, abundant cytoplasm, and prominent perinuclear cytoplasmic hoffs. These cells also expressed detectable, albeit low, Tcl1 by Western blot (4.2% of naïve B levels). Overall, Western blot analysis of isolated B cell subsets refined the developmental pattern of Tcl1 expression and showed that expression is progressively down-regulated during final B cell maturation in lymphoid tissues.
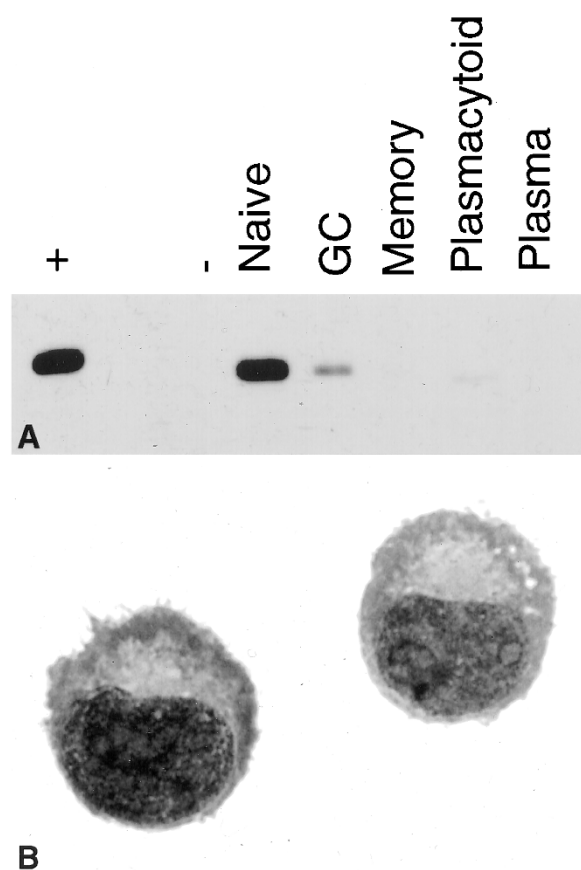

Figure 2.

Tcl1 protein expression varies with B cell developmental stage. A, Anti-Tcl1 Western blot analysis of isolated B cell populations from human tonsil. P3HR-1 (+) and BCBL-1 (-) control lysates are also shown. The level of Tcl1 expression seen in naïve B cells (>90% pure, CD10+/IgD+) was determined by densitometry and set arbitrarily at 100%; this level is comparable to the P3HR-1 BL tumor cell line. Germinal center (GC) B cells (>85% pure, CD10+/IgD-) express 19% of the naïve B cell level, whereas memory B cells (>95% pure, CD10-/CD19+/IgD-) and plasma cells (CD138+) do not express Tcl1. Plasmacytoid B cells, grown on CD40L+ feeder cells supplemented with IL4 and IL10, express 4% of the naïve B cell level of Tcl1. B, Wright-Giemsa stain of plasmacytoid B cells demonstrating characteristic morphologic features, including eccentric nuclear placement, abundant cytoplasm, and perinuclear hoff.

Tcl1-Expression in Patient-Derived B Cell Lymphomas Also Depends on Developmental Stage

Because EBV does not affect normal B cell expression of Tcl1, we investigated whether B cell lymphomas from immune-competent individuals retain Tcl1 expression in a pattern reflective of their developmental derivation. In this case, Tcl1 expression would represent a useful marker for the stage of differentiation of neoplastic B cells, particularly between GC and post-GC stages of development. In addition, *TCL1* has known tumor-promoting capabilities in T cells and its expression may be indicative of a growth or survival advantage that may correlate with clinical aggressiveness, outcome, or mode of therapy in B cells. The results from Table 1 show that *TCL1* is abundantly expressed in GC-derived BL but not in post-GC–derived myeloma or PEL cell lines. To determine whether these cell line expression results are similar to results obtained from patients, we assessed the Tcl1 expression pattern in a large number and wide variety of tumor samples (Fig. 3 and Table 2).

In immune-competent individuals, the data show a marked transition in Tcl1 expression between naïve/GC and post-GC B cell lymphomas (Hummel et al, 1994). In 23 of 27 (85%) cases naïve/GC-derived B lymphomas expressed abundant Tcl1, whereas only 16 of 41 (39%) cases of post-GC B lymphomas were
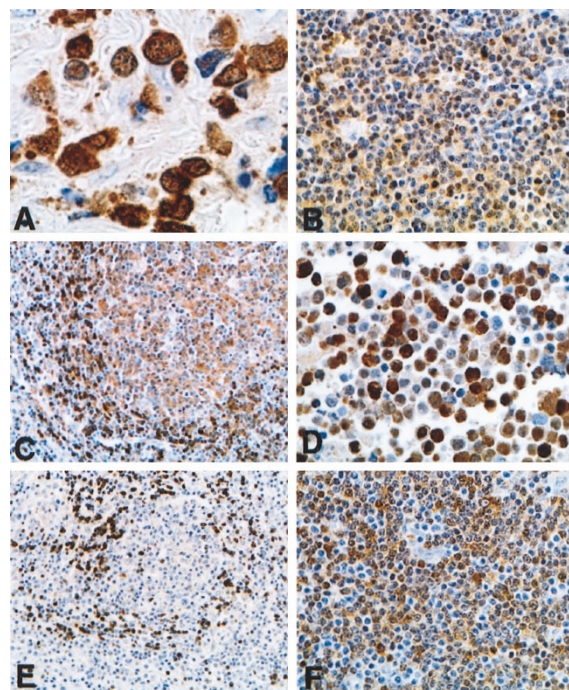

Figure 3.

Immunohistochemical staining of patient B cell tumor samples with Tcl1 antisera. A, Lymphoblastic lymphoma, pre-B cell type, with strong 3+ staining for Tcl1. B, Mantle cell lymphoma with 2+ staining for Tcl1. C, Follicle center cell lymphoma with 2+ staining of the neoplastic follicle. The surrounding benign mantle cells are 3+ positive. D, DLBCL with 3+ staining for Tcl1. E, Marginal zone lymphoma negative for Tcl1. Strong staining is present in the residual mantle cells, which surround a follicle that has been colonized or replaced by a Tcl1-negative lymphoma. F, Marginal zone lymphoma with 2+ staining for Tcl1. Hematoxylin counterstain. Original magnifications: A, ×600; B, C, D, and F, ×250; E, ×120.

Table 2. Tcl1 Protein Expression Levels in Patient-Derived B and T Cell Lymphomas[a]

Tumor Classification	Tcl1+ Cases/No. of Cases	Tumor Cells + (%)	Relative Level of Tcl1 Expression
Pre-B lymphoma			
Lymphoblastic	3/3	90	3+
Naïve/GC-derived B cell lymphoma			
Mantle cell	12/14	70–95	2+ to 3+
Follicular grade I	7/7	60–90	1+ to 3+
Follicular grade II/III	3/5	80–100	1+ to 3+
Burkitt lymphoma	1/1	90	3+
Post GC-derived B cell lymphoma			
Marginal zone	4/10	5–80	2+ to 3+
Diffuse large B cell (DLBCL)			
HIV-seronegative	12/31	< 5–90	1+ to 3+
HIV-seropositive (AIDS)	1/1	90	3+
Lymphoplasmacytic lymphoma	0/3	0	0
Plasma cell myeloma	0/3	0	0
Heterogeneous/uncertain derived B cell lymphoma			
SLL (60% pre-GC, 40% post-GC)	10/11	10–70	1+ to 3+
AIDS PEL	0/2	0	0
T cell lymphoma			
Anaplastic large T cell	0/1	0	0
Cutaneous T cell	0/4	0	0
Mycosis fungoides	0/2	0	0
Peripheral large T cell	0/4	0	0
T/NK	0/2	0	0
Hodgkin disease			
NLPHD, NSHD	0/10	0	0

SLL, small lymphocytic lymphoma; PEL, primary effusion lymphoma; T/NK, natural killer cell, T cell type; NLPHD, nodular, lymphocyte-predominant Hodgkin disease; NSHD, nodular sclerosing Hodgkin disease.

[a] Formalin-fixed, paraffin-embedded tissues were stained with Tcl1 antisera by immunohistochemical methods as previously described (Said et al, 1997, 1998; Teitell et al, 1999). Cell staining intensity was scored visually from 1+ to 3+ (see Fig. 3 for examples). Grouping of B cell lymphoma origins was based on published reports (Kuppers et al, 1999). SLL are considered both pre- and post-GC in derivation. The histogenesis of AIDS PEL is not fully resolved (Matolcsy et al, 1998).

Tcl1-positive. Three cases of pre-B lymphoblastic lymphoma strongly expressed Tcl1, consistent with earlier results showing that Tcl1 is highly expressed in pre-B and immature B lymphocytes (Virgilio et al, 1994). If cases with less than 10% of cells staining for Tcl1 are considered negative, due to potential residual or infiltrating normal B lymphocytes, then the number of post-GC positive cases drops to 10 of 41 (24%), whereas the number of naïve/GC positive cases remains the same. The expression of Tcl1 is statistically different between GC and post-GC derived B cell lymphomas in immune-competent individuals ($p < 0.001$). Finally, one post-GC AIDS-DLBCL patient sample stained strongly (3+) for Tcl1 and was not included in these figures. If this case is added to previously reported results on *TCL1* expression in systemic AIDS-DLBCL, then 7 of 9 (78%) such cases now surveyed express *TCL1* (Teitell et al, 1999).

Tcl1 and Bcl-6 Expression Patterns Are Similar in DLBCL from Patient Samples

Bcl-6 normally is expressed in GC B cells but not in post-GC B cells, whereas Tcl1 is expressed from early B cell development in the bone marrow through GC stages of development (Cattoretti et al, 1995; Onizuka et al, 1995). Although distinct, these expression patterns are similar in that a concordant down-regulation of expression of these two oncoproteins occurs in post-GC B cells. To investigate the persistence of a concordant Tcl1 and Bcl-6 expression pattern in post-GC B cell tumors, 10 DLBCL (five Tcl1+ and five Tcl1-) were selected and stained with Bcl-6 antibody. As summarized in Table 3, 2 of 10 (20%) cases expressed Bcl-6 but not Tcl1, whereas 3 of 10 (30%) expressed neither, and 5 of 10 (50%) expressed both oncoproteins. There were no cases in which Tcl1 was positive and Bcl-6 was negative. In those cases where both proteins were expressed, the proportion of tumor cells expressing Bcl-6 was equivalent or higher than the proportion of tumor cells expressing Tcl1, whereas the levels of expression were often similar. These results show that Tcl1 and Bcl-6 expression are concordant in 8 of 10 (80%) DLBCL tested, which is consistent with the expression pattern of both genes in nonmalignant GC and post-GC B lymphocytes. However, concordant expression of these two oncoproteins does not necessarily indicate a mechanistic regulatory linkage between *TCL1* and *BCL-6* oncogenes.

Discussion

In this study we have shown that the transition in Tcl1 expression that occurs in normal peripheral B lympho-

Table 3. Relative Tcl1 Versus Bcl-6 Oncoprotein Expression Levels in DLBCL[a]

Case No.	Tumor Cells Tcl1 + (%)	Level of Tcl1 Expression[b]	Tumor Cells Bcl-6 + (%)[c]	Level of Bcl-6 Expression
1	< 5	1+	60	1+ to 3+
2	60	2+	90	3+
3	0	0	95	3+
4	< 10	1+	80	2+
5	0	0	30	2+
6	0	0	0	0
7	40	1+	70	2+
8 (AIDS)[d]	60	2+	80	3+
9	0	0	0	0
10	0	0	0	0
11	10	1+	90	3+

[a] Formalin-fixed, paraffin-embedded tissues were stained with Tcl1 antisera and Bcl-6 antibody.
[b] Cell staining intensity was scored from 1+ to 3+.
[c] Bcl-6 staining was performed on five Tcl1+ and five Tcl1− cases due to tissue availability.
[d] Case 8 is from a patient with an AIDS-DLBCL.

cyte development usually is maintained in B cell lines and B lymphoma patient samples derived from different stages of B cell maturation. However, this correlation is not maintained for AIDS-DLBCL, because our data show that a markedly higher proportion of AIDS-DLBCL expressed Tcl1 than did DLBCL from immune-competent individuals. We also observed a concordant expression pattern between Tcl1 and Bcl-6 oncoproteins in a majority of DLBCL patient samples tested. In contrast to previous results, we found no association between Tcl1 expression and EBV involvement in BL cell lines and we showed that functional maturity of the immune system is unimportant for peripheral Tcl1 expression. Finally, we demonstrated that PEL resemble myelomas in their lack of Tcl1 expression. Our findings suggest a useful role for Tcl1 detection in determining the developmental derivation of mature, peripheral B cell lymphomas, except in AIDS-DLBCL where discordant expression has a potential etiologic role.

TCL1 expression did not differ significantly in three paired samples of EBV-positive and negative BL cell lines and expression was consistently present at high levels in EBV-immortalized PBC and fetal cord cells. MACS sorting results presented here, combined with earlier immunohistochemical results, clearly show that Tcl1 levels reflect the stage of development of B cells in peripheral lymphoid tissues, with expression declining in GC B cells from a peak in noncycling mantle zone B cells to no expression in post-GC B cells. This same pattern of expression also is seen in B cell lymphomas from immune-competent individuals. However, about 25% of those with DLBCL still express Tcl1, suggesting perhaps that either these DLBCL derive from GC B–like cells (see below) or that in one-quarter of DLBCL samples Tcl1 expression is dysregulated by an unknown mechanism. In contrast to DLBCL, *TCL1* expression in AIDS-DLBCL persists, suggesting that either down-regulation of *TCL1* is dependent on normal immune functions and that *TCL1* is expressed aberrantly in the majority of AIDS-

DLBCL or that, unlike DLBCL, most AIDS-DLBCL derive from GC B–like cells.

Recently, a new approach for classifying DLBCL was reported (Alizadeh et al, 2000). A model was proposed for the developmental origins of DLBCL based on gene chip expression profiling. Tcl1 expression by immunohistochemistry in 31 independent DLBCL samples shown here is strikingly consistent with the *TCL1* results obtained for 46 different DLBCL by gene profiling. We find 12 of 31 (39%) (or 8 of 31 [26%] if cases with less than 10% Tcl1-positive cells are considered as negative) of DLBCL express Tcl1, whereas the gene profiling study detected 14 of 46 (30%) of the DLBCL samples expressed *TCL1* above a pooled sample background. However, gene profiling is unable to detect variable levels of expression of *TCL1* within tumor and normal cells of a given sample, whereas our study demonstrates a wide range of Tcl1 expression in cells from different samples. One disadvantage of gene profiling is that the averaging of marker gene expression within a given sample can mask the sample extremes.

From gene profiling, DLBCL can be divided into two subgroups: GC B–like DLBCL and activated B–like DLBCL (Alizadeh et al, 2000). One of the key markers used to divide tumors into these two subgroups is *BCL-6*. Our results show several distinct patterns of Tcl1 and Bcl-6 expression in DLBCL. The lack of expression of both Tcl1 and Bcl-6 suggests a post-GC, preterminal differentiation stage of derivation, whereas the coexpression of both proteins is consistent with a GC derivation. However, it must be noted that the patterns of expression for Tcl1 and Bcl-6 in GC are distinct, with Bcl-6 staining almost all GC cells and Tcl1 staining decreased from high levels in the mantle zone to low levels in most GC cells. The observation that some tumors express Bcl-6 and not Tcl1 suggests a potential intermediate cell derivation, possibly from a unique Bcl-6 positive, Tcl1 negative GC cell stage of development. Alternatively, perhaps this pattern represents an aberration in the normal

expression of one of these two genes caused by the tumorigenic process. Interestingly, when the gene expression profiles for *BCL-6* and *TCL1* are compared, it appears that *TCL1* may be superior to *BCL-6* for distinguishing naïve (high), GC (low), and post-GC (negative) stages of B lymphoma derivation (Alizadeh et al, 2000). Tcl1 immunohistochemistry clearly provides a useful tool to delineate naïve/GC from post-GC B cell lymphomas in immune-competent individuals and may augment subclassification based on Bcl-6 reactivity. The dissection of DLBCL into Tcl1 positive and negative categories may also have important implications for the biology and treatment of this aggressive lymphoma subtype.

In this and an earlier study, we have shown that the percentage of AIDS-DLBCL expressing *TCL1* (7 of 9, 78%) more closely resembles the percentage of naïve/GC lymphomas that express Tcl1 (23 of 27, 85%) than the proportion of DLBCL expressing Tcl1 from immune-competent individuals (12 of 31, 37%, or 8 of 31, 26%). This occurrence may explain the ease of originally isolating the *TCL1* oncogene with subtractive techniques because there appears to be a significant difference in expression between AIDS-DLBCL and DLBCL patient tumor samples (Teitell et al, 1999). This difference may reflect the disruption of normal lymphoid architecture present in late-stage AIDS patients (reviewed in Gaidano et al, 1998; Hamilton-Dutoit et al, 1991; Herndier et al, 1994; Knowles, 1997; Raphael et al, 1991, 1994). Interestingly, two recent studies disagree on the proportion of DLBCL from immune-competent patients that express Tcl1 (Nakayama et al, 2000; Narducci et al, 2000). One study showed 18%, the other showed 60%, of DLBCL expressed Tcl1. Our results support the lower figure in DLBCL from immune-competent individuals and the higher figure in immune-deficient patients. Explanations for this discrepancy might include differential application of the criteria used to diagnose DLBCL because of tumor heterogeneity or unknown influences from distinct patient populations. DLBCL are a heterogeneous group with variable treatment outcomes and prognoses, and the heterogeneity in Tcl1 expression demonstrated here may have clinical relevance. Moreover, the relatively high proportion of immune-deficient AIDS-DLBCL that express Tcl1 compared with immune-competent DLBCL may have a bearing on the pathogenesis of these neoplasms.

PEL and myeloma have in common a complete lack of Tcl1 expression in all cell lines and patient samples examined thus far. The derivation of myeloma from an end-stage B-lineage plasma cell is well documented. However, there is no consensus on the stage or stages of B cell development that give rise to PEL in immune-compromised individuals (Matolcsy et al, 1998). Our finding of complete absence of Tcl1 in PEL adds some support to an origin from post-GC through terminally differentiated B lymphocytes, similar to myeloma. In this regard, the BC-3 PEL line stands out as a possible exception to this hypothesis because it is considered to arise from a pre-GC stage of development due to a lack of somatic hypermutation. How-

ever, BC-3 expresses multiple late lymphocyte activation and adhesion markers, including CD30, CD38, CD138, HLA-DR, and CD54, which is consistent with a GC or post-GC derivation (Drexler et al, 1998). It appears, therefore, that most PEL derive from GC or post-GC B cell precursors.

Materials and Methods

EBV Immortalization and Cell Culture

Peripheral blood lymphocytes and fetal cord cells were harvested from HIV-seronegative individuals and immortalized by incubation with supernatant from the EBV-producing marmoset cell line B95-8 according to standard protocols (Miller and Lipman, 1973; Miller et al, 1974; Ounanian et al, 1992). All cell lines, except PEL lines, were maintained at 37° C in 5% CO_2 and grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FBS. The PEL lines were grown in medium containing 20% serum. EBV-immortalized cell lines were weaned from 20% to 10% FBS after cell line expansion. Cell lines were provided as kind gifts from Drs. B. Bonavida (2F7 and R), S. Plaeger-Marshall (immortalized fetal cord and PBC lines), O. Martinez-Maza (P3HR-1), and R. Sun (BL41, BL41/B95-8, BJAB, and BJAB-B1; University of California at Los Angeles School of Medicine, Los Angeles, California) and from Dr. J. T. Sample (Akata A1.5, Akata 2A8, and Akata 3F2; University of Tennessee Health Sciences Center, Memphis, Tennessee).

Northern Blot Analysis

Total cellular RNA was isolated (Rneasy Mini Kit; Qiagen, Santa Clarita, California) and 10 μg per sample was size-fractionated on a 1% denaturing formaldehyde gel. Size-fractionated RNA was transferred to a nitrocellulose membrane (Nitroplus; MSI, Westborough, Massachusetts) in 10× SSC and fixed in place by baking at 80° C for 1 hour in a vacuum oven. Fifty nanograms of a 350-bp human *TCL1* cDNA fragment were random-primed (Prime-It II; Stratagene, La Jolla, California) with α-P^{32} dATP for use as a probe. Membranes were prehybridized at 42° C for 2 to 4 hours in Denhardt's solution containing 2.5 mg boiled herring-sperm DNA, and then hybridized with 10^6 counts/ml radiolabeled *TCL1* probe at 42° C overnight. Hybridized membranes were washed stringently (final wash: 0.1× SSC/0.1% SDS, 30 minutes, 52° C) and *TCL1* RNA was detected by autoradiography and by Phosphorimager analysis with the ImageQuant program (Molecular Dynamics, Sunnyvale, California). Equal RNA loading was assured by ultraviolet visualization of ethidium bromide stained gels (Fig. 1).

Patient Tumor Samples and Immunohistochemistry

Malignant lymphomas were characterized by immunophenotype, classified according to the World Health Organization classification system, and retrieved from the files of the surgical pathology division of the Anatomic Pathology Department at University of Cal-

ifornia at Los Angeles (Harris et al, 1997). Serial paraffin sections were stained with Tcl1 antisera and Bcl-6 antibodies (DAKO, Carpinteria, California) using immunoperoxidase techniques as previously described (Said et al, 1997, 1998; Teitell et al, 1999). Results were interpreted according to the percentage of neoplastic cells staining positive for Tcl1 or Bcl-6 together with the intensity of staining (1 to 3+). Negative controls consisted of substituting the primary Tcl1 antisera with preimmune serum or substituting the primary Bcl-6 antibody with a non-cross-reactive isotype-specific monoclonal antibody. Positive controls, consisting of sections of hyperplastic tonsil, were run with all cases. Fresh-frozen cell line pellets were stained for TCL1 expression by a similar immunoperoxidase technique (not shown).

B Lymphocyte Fractionation from Human Tonsil

Hyperplastic tonsils were obtained from the Human Tissue Resource Center at University of California at Los Angeles with strict adherence to established guidelines for the protection of human subjects (IRB). B cell subsets were then isolated as previously described (Babcock and Thorley-Lawson, 2000). Tonsils were minced under sterile conditions in 1× PBS (pH 7.4, 1% BSA, and 5 mM EDTA) and passed through a 40-μm filter to remove connective tissue and debris. Viable lymphocytes were isolated by centrifugation on a Ficoll-Paque Plus cushion (Amersham Pharmacia Biotech, Piscataway, New Jersey), followed by washing in PBS. B cells were fractionated using a MACS system (Miltenyi Biotec, Auburn, California) according to the manufacturer's instructions. Isolated populations were analyzed by flow cytometry to determine percentage purity. Naïve B cells (CD3-/IgD+) were isolated by negative selection with anti-CD3 antibody (FITC-CD3, clone S4.1; BD PharMingen, San Diego, California) followed by MACS positive selection with anti-IgD antibody (R-PE-IgD, clone 1A6-2; BD PharMingen) to more than 90% purity. GC B cells (CD3-/CD10+/IgD-) were isolated by MACS negative selection with anti-CD3 antibody (R-PE-CD3, clone 5-1B4; Caltag, Burlingame, California) and anti-IgD antibody (BD PharMingen), followed by MACS positive selection with anti-CD10 antibody (R-PE-CD10, clone 5-1B4; Caltag) to more than 85% purity. Memory B cells (CD3-/CD10-/IgD-) were isolated by MACS negative selection with anti-CD3 antibody (Caltag) and anti-IgD antibody (BD PharMingen), followed by MACS negative selection with anti-CD10 antibody (Caltag) to more than 95% purity. Memory B cells were verified by additional staining and flow cytometry with anti-CD19 antibody (FITC-CD19, leu-12; BD PharMingen) and anti-CD20 antibody (FITC-CD20, leu-16; BD PharMingen). Plasma cells (CD3-/CD138+) were isolated as previously described (Jego et al, 1999) by MACS negative selection with anti-CD3 antibody (BD PharMingen) followed by MACS positive selection with anti-CD138 antibody (R-PE-CD138, clone BB4; BD PharMingen).

In Vitro Generation of Plasmacytoid B Cells

Plasmacytoid B cells were generated as previously described (Fluckiger et al, 1998). Briefly, after tonsil disruption and Ficoll-Paque Plus centrifugation, B lymphocytes were positively selected with anti-CD19 microbeads (Miltenyi Biotec) using MACS. Isolated CD19+ B cells (8 × 10^5 cells/well) then were incubated in 12-well tissue culture plates on irradiated (8,000 rad) CD40L-expressing L cell fibroblasts (approximately 60% confluent) in RPMI 1640 medium containing 20% FCS, 20 ng/ml IL4, 20 ng/ml IL10, and antibiotics. IL4 and IL10 were resupplemented on Day 4 of culture. After one week, cells were harvested and analyzed by anti-Tcl1 Western blot and plasmacytoid features confirmed with Wright-Giemsa staining of cytospin pellets.

Anti-Tcl1 Western Blot Analysis

Fractionated B cells were washed twice with PBS then lysed in NP-40 lysis buffer (50 mM Tris, pH 7.6, 300 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, and protease inhibitors) and centrifuged at 15,000×*g* for 20 minutes at 4° C. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, California). P3HR-1 cell lysates were used as a Tcl1-positive control and BCBL-1 cell lysates were used as a negative control. Six micrograms of total protein for each sample was separated by 15% SDS PAGE and transferred to a nitrocellulose membrane (Micron Separations, Westborough, Massachusetts). The membrane was blocked in Tris buffered saline plus Tween (TBST, TBS, and 0.1% Tween) and 5% dry milk for 15 minutes. The membrane then was incubated with Tcl1 antisera (Teitell et al, 1999) in TBST (diluted 1:7,500) for 1 hour, then washed for 3 minutes 6 times in TBST. After this, the filter was incubated in antirabbit IgG HRP-linked antisera (New England Biolabs, Beverly, Massachusetts) in TBST (diluted 1:5,000) for 1 hour, followed by 6 washes in TBST and developed with ECL+Plus (Amersham Pharmacia Biotech). Densitometric analysis was performed using the ChemiImager 5500 with Alpha-EaseFC software (Alpha Innotech, San Leandro, California).

Statistical Analysis

The null hypothesis, that Tcl1 expression is equivalent in naïve/GC and in post-GC–derived B cell lymphomas, was evaluated with a two-sample proportion test.

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