TORC2 regulates germinal center repression of the TCL1 oncoprotein to promote B cell development and inhibit transformation

Ali I. Kuraishy*, Samuel W. French†, Mara Sherman‡, Marco Herling§, Dan Jones§, Randolph Wall‡¶‖, and Michael A. Teitell†‡‖**

Departments of *Human Genetics, †Pathology and Laboratory Medicine, and ¶Microbiology, Immunology, and Molecular Genetics, ‡Molecular Biology Institute, and ‖Institute for Stem Cell Biology and Medicine and Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA 90095; and §Department of Hematopathology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030

Aberrant expression of the TCL1 oncoprotein promotes malignant transformation of germinal center (GC) B cells. Repression of *TCL1* in GC B cells facilitates FAS-mediated apoptosis and prevents lymphoma formation. However, the mechanism for this repression is unknown. Here we show that the CREB coactivator TORC2 directly regulates *TCL1* expression independent of CREB Ser-133 phosphorylation and CBP/p300 recruitment. GC signaling through CD40 or the BCR, which activates pCREB-dependent genes, caused TORC2 phosphorylation, cytosolic emigration, and *TCL1* repression. Signaling via cAMP-inducible pathways inhibited *TCL1* repression and reduced apoptosis, consistent with a prosurvival role for TCL1 before GC selection and supporting an initiating role for aberrant *TCL1* expression during GC lymphomagenesis. Our data indicate that a novel CREB/TORC2 regulatory mode controls the normal program of GC gene activation and repression that promotes B cell development and circumvents oncogenic progression. Our results also reconcile a paradox in which signals that activate pCREB/CBP/p300 genes concurrently repress *TCL1* to initiate its silencing.

gene regulation | lymphomagenesis | signal transduction

The germinal center (GC) is home to T cell-dependent antigen-driven B cell maturation, memory B and plasma cell production, and the site of origin for most human B cell lymphomas (1–3). GC B cells undergo critical changes in gene expression that are required for proper development and protection from oncogenesis (4, 5). The generation of an appropriate humoral response is insured by negative selection, primarily mediated by FAS-induced apoptosis (6–8). Escape from apoptosis results in autoimmunity and B cell transformation (8).

TCL1 functions as a coactivator of the cell survival kinase AKT (9, 10). In mature T cell tumors, *TCL1* expression is aberrantly elevated by rearrangements into T cell receptor loci (11). Physiologic *TCL1* expression is largely limited to B lineage cells and is robust from pre-B cells through peripheral naïve B cells, followed by a critical 40–60% repression in GC B cells and complete silencing in post-GC memory B and plasma cells (12, 13). Most B cell lymphomas that arise by transformation of GC-experienced B cells exhibit elevated *TCL1* expression by escape from GC mechanism(s) of *TCL1* repression (12, 14, 15). Interestingly, transgenic mice with *TCL1* expression levels stabilized in GC lymphocytes develop cancers that resemble a spectrum of human GC B cell lymphomas (16).

Unrepressed *TCL1* expression inhibits FAS-induced B cell apoptosis independent of activation by BCR survival signaling (17, 18). Impaired apoptosis from failed *TCL1* repression in GC B cells connects *TCL1* dysregulation in patient lymphomas (12, 14) to a mechanism for increased transformation (16, 18). Factors so far suggested to regulate *TCL1*, including Nur77 (19, 20), *miRNA-29* and *miRNA-181* (21), Sp1 (22), and EBV infection (23), fail to adequately explain *TCL1* repression in GC B cells and its continued aberrant expression in lymphomas (11).

Its role in promoting B cell development and preventing lymphomagenesis makes *TCL1* a highly important direct target of the regulatory program that controls a battery of GC B cell genes, although the regulatory program controlling *TCL1* is unknown (24). Therefore, we focused on the mechanism(s) of *TCL1* regulation, especially during GC B cell processes, as a strategy for identifying new regulatory programs that control key GC B cell genes and for discovering key sites of *TCL1* dysregulation in B cells that could promote malignant transformation.

Results

CREB Controls *TCL1* Expression. DNase I footprint and MatInspector revealed a CREB response element (CRE)-like half-site (GACGT) within the *TCL1* promoter [supporting information (SI) Fig. 7A] (25). Electrophoretic mobility shift assays using B cell nuclear extracts, CREB-specific antiserum, and purified CREB-1 protein demonstrated that CREB bound this CRE half-site (SI Fig. 7 B–E). To provide physiologic relevance, ChIP studies demonstrated CREB and pCREB associated with the endogenous *TCL1* promoter in *TCL1* expressing Nalm-6 pre-B and Ramos B cells (SI Fig. 8). The effect of CREB on *TCL1* promoter activity was determined in HEK293T cells using 424 bp of the *TCL1* promoter (−424luc), as done before (SI Fig. 9) (22). *TCL1* is not expressed in HEK293T cells, although its promoter is highly active in transient reporter assays (22). CREB transactivation is mediated by increased cAMP, which results in protein kinase A or PKC activation and phosphorylation of CREB on Ser-133 (pCREB-133) (26–28). pCREB-133 recruits CBP (CREB-binding protein) or its paralogue, p300, to activate CREB-responsive promoters (27). Incubation with dibutyryl cAMP (dbcAMP) resulted in a 3-fold induction, whereas mutation of the CRE half-site (GACGT→GATCT) to block CREB binding abolished cAMP responsiveness. However, a *TCL1* promoter response to cAMP could still be CREB-independent (27). Cotransfection of a CREB expression plasmid with the −424luc reporter resulted in a 2.5-fold induction, whereas cotransfection with the −m424luc reporter failed to activate *TCL1*, indicating that cAMP and CREB transactivation was direct at the CRE half-site. CREB shRNA validated a role for

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Fig. 1. cAMP increases *TCL1* expression in B cells and patient B-CLL samples. (*A*) Ramos B cells were incubated with 0.1 mM dbcAMP for the times indicated, and whole-cell lysates were immunoblotted with CREB or pCREB-133 Abs. (*B*) *TCL1* mRNA levels in Ramos cells responding to dbcAMP for the indicated times were measured by real-time quantitative RT-PCR (SYBRgreen), with expression normalized to a *36B4* gene control. (*C*) Immunoblot for *TCL1* and β-actin in Ramos cells responding to dbcAMP for the indicated times. Band ratios were determined with densitometry. (*D*) −424luc reporter activity in three different patient B-CLL samples stimulated with 0.1 mM dbcAMP for 72 h. Data are representative of two independent experiments.

CREB in regulating *TCL1*, with knockdown to 25% of native levels resulting in robust repression of *TCL1* promoter activity. Overall, the data indicate that CREB along with Sp1 supports robust basal activity of the *TCL1* promoter (22).

We next examined a role for cAMP in regulating endogenous *TCL1* in B cells. Ramos B cells treated with dbcAMP showed a modest increase in pCREB-133 at 30 min that returned to baseline after 1 h (Fig. 1*A*), paralleling a 2-fold increase in *TCL1* mRNA (Fig. 1*B*). After 6 h of dbcAMP, *TCL1* expression returned to baseline, duplicating the induction by dbcAMP in transient reporter assays (SI Fig. 9*B* and data not shown). Significantly, TCL1 protein increased between 6 and 24 h (Fig. 1*C*), with an ≈5-fold increase at 24 h. dbcAMP treatment of Jurkat T cells, which do not express *TCL1*, failed to induce *TCL1* (data not shown), likely because dbcAMP cannot overcome epigenetic silencing at the *TCL1* locus (22). In contrast, B-chronic lymphohocytic leukemia (B-CLL) cells likely require *TCL1* for survival (12, 14, 29, 30), and the −424luc reporter was activated by dbcAMP in at least two of three patient samples between 14- and 140-fold over untreated levels (Fig. 1*D*). Combined, these data indicate that cAMP induces TCL1 in GC-derived B cell lines and primary B-CLL samples, supporting physiologic and pathologic roles for *TCL1* regulation *in vivo*.

TORC2 Controls *TCL1* Promoter Activity. Our data predict that cAMP-mediated protein kinase A or PKC activation led to CREB Ser-133 phosphorylation, promoter binding, and CBP/p300 coactivator recruitment as a mechanism for *TCL1* expression. To examine this idea further, a dominant-negative mutant CREB (mCREB) construct was cotransfected with −424luc or −m424luc reporters into HEK293T cells. mCREB contains a Ser-133→Ala-133 substitution, blocking CREB Ser-133 phosphorylation and activation of the canonical CREB transactivation pathway without blocking CREB binding to CRE-containing promoters (27). Because CREB knockdown repressed basal *TCL1* promoter activity (SI Fig. 9 *D* and *E*), coexpression of mCREB was expected to also decrease *TCL1* promoter activity (27). Unexpectedly, mCREB expression resulted in an ≈2-fold induction of −424luc reporter activity over robust unstimulated levels (Fig. 2*A*). mCREB did not appreciably affect −m424luc reporter activity (<2-fold change), supporting the specificity of mCREB for the CRE half-site in the *TCL1*
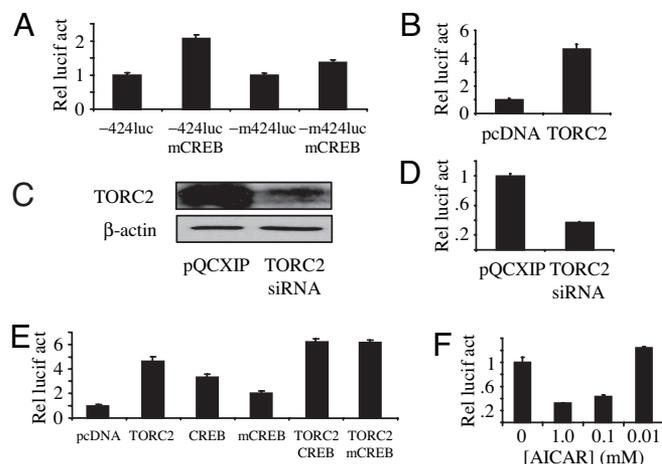

Fig. 2. TORC2-dependent, pCREB-133-independent *TCL1* activation. (*A*) −424luc or −m424luc reporter activity in HEK293T cells 48 h after transfection, with or without cotransfection of the mCREB (Ser-133→Ala-133) expression construct. (*B*) −424luc reporter activity in HEK293T cells 48 h after transfection, with or without cotransfection of the psportTORC2 (M. Montminy) expression construct. (*C*) Immunoblot for TORC2 repression in HEK293T cells 48 h after transfection with a TORC2 siRNA expression construct. (*D*) −424luc reporter activity in HEK293T cells at 48 h with or without cotransfection of a TORC2 siRNA expression construct. (*E*) −424luc reporter activity in HEK293T cells at 48 h with or without cotransfection of TORC2, CREB, or mCREB expression constructs. (*F*) −424luc reporter activity in HEK293T cells 48 h after transfection, with or without the indicated concentrations of AICAR added 6 h before assay harvest. For all experiments the data were normalized to a cotransfected *Renilla* luciferase (pRL*CMV* luciferase) reporter construct. Data represent the mean ± SD of two independent experiments, each done in triplicate.

promoter. Unexpectedly, the data suggest that pCREB-133 is dispensable for CREB activation of the *TCL1* promoter.

An alternative mechanism for CREB control of *TCL1* expression was next considered. A leading candidate for testing was the transducer of regulated CREB (TORC) protein. TORC proteins bind to CREB and activate target genes independent of pCREB-133 (31). TORC1 had no effect on the −424luc reporter (data not shown), consistent with the lack of TORC1 expression in human B cells (ref. 31 and data not shown), whereas TORC2 expression activated −424luc ≈5-fold (Fig. 2*B*). HEK293T cells, which express endogenous TORC2 (Fig. 2*C*), were cotransfected with TORC2 siRNA and the −424luc reporter, resulting in decreased *TCL1* promoter activity to ≈30% of control levels (Fig. 2*D*), which was similar to the repression detected with CREB shRNA (SI Fig. 9*E*). When coexpressed, CREB and TORC2 increased *TCL1* promoter activity additively by >6-fold (Fig. 2*E*). An identical induction of the −424luc reporter was also achieved by using mCREB with TORC2 cotransfection, further demonstrating the pCREB-133-independent, TORC2-dependent transactivation of *TCL1*.

Incubation with 5-aminoimidazole-4-carboxamide riboside (AICAR, an AMP analogue) results in TORC2 Ser-171 phosphorylation (pTORC2–171) and translocation out of nucleus into the cytoplasm, thereby repressing TORC2-dependent target genes (32). Because *TCL1* promoter activity depended on TORC2 expression and not pCREB-133, we postulated that AICAR would repress *TCL1*. Consistent with this notion, AICAR caused a dose-dependent repression of the *TCL1* promoter to ≈30% of untreated control cells (Fig. 2*F*). These data indicate that CREB and TORC2 transactivate *TCL1* and that unstimulated, or robust basal, *TCL1* expression depends on these interacting transfactors.
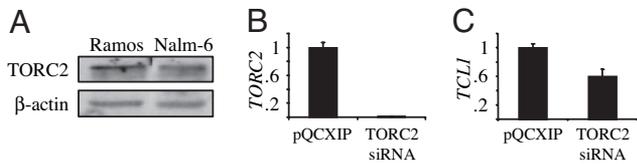
Fig. 3. TORC2 inhibition represses *TCL1* expression in Nalm-6 pre-B cells. (*A*) Immunoblot showing relative expression levels of TORC2 in Ramos B cells and Nalm-6 pre-B cells. (*B*) Real-time quantitative RT-PCR of *TORC2* mRNA levels in polyclonal Nalm-6 cells stably expressing *TORC2* siRNA. (*C*) Real-time quantitative RT-PCR of *TCL1* mRNA levels in polyclonal Nalm-6 cells stably expressing *TORC2* siRNA. Data are representative of two independent experiments and plot the mean ± SD of triplicate measurements.

TORC2 Regulates *TCL1* Expression in B Cells. Western blot demonstrated abundant TORC2 in Nalm-6 pre-B and Ramos B cells (Fig. 3*A*). siRNA knockdown reduced TORC2 expression in Nalm-6 cells by ≈99% (Fig. 3*B*), whereas Ramos cells did not tolerate multiple TORC2 knockdown strategies (data not shown). TORC2 reduction resulted in a 40% repression in endogenous *TCL1* expression in Nalm-6 cells (Fig. 3*C*). These data support transient *TCL1* reporter results in HEK293T cells (Fig. 2) and also recapitulate the extent of *TCL1* repression detected in primary GC B cells (11), suggesting a central role for TORC2 in regulating endogenous *TCL1* expression in GC B cells.

BCR and CD40L Signaling Mediate TCL1 Repression Independent of CREB Phosphorylation. Based on these findings, we postulated that CREB or TORC2 inhibition could repress *TCL1* in GC B cells (12, 15). To test this idea, Ramos B cells were stimulated in a manner that resembled a T-dependent GC reaction by signaling through CD40 and the BCR (18). Ramos B cells undergo BCR-mediated apoptosis and provide a model for deletion of self-reactive GC B cells (33). BCR engagement results in CREB phosphorylation (28), indicating potential relevance for CD40 and BCR signaling in regulating *TCL1*. First, pCREB-133 was determined with anti-IgM to establish that BCR signaling was intact in Ramos cells. As expected, anti-IgM resulted in a significant increase in pCREB-133 that was enhanced by coaddition of cAMP without a change in total CREB protein levels (Fig. 4*A*). Surprisingly, *TCL1* mRNA expression was reduced by ≈80% at 12 h of anti-IgM stimulation (Fig. 4*B*). TCL1 protein expression was also markedly reduced after 16–20 h of anti-IgM (Fig. 4*C*). These data support the −424luc reporter results,
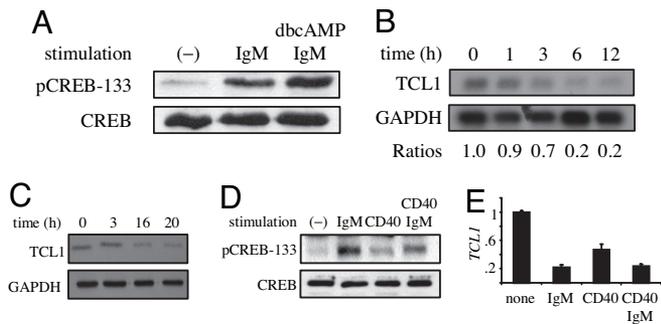

Fig. 4. BCR or CD40 signaling activates CREB and represses TCL1 expression. Ramos B cells were treated with 10 μg/ml anti-IgM, 1 μg/ml anti-CD40, or 0.1 mM dbcAMP for the times indicated. (*A*) Immunoblot for CREB or pCREB-133 at 1 h of stimulation. (*B*) Northern blot for *TCL1* and *GAPDH* with densitometry quantification. (*C*) Immunoblot for TCL1 and β-actin. (*D*) Immunoblot for CREB or pCREB-133 at 1 h of stimulation. (*E*) Real-time quantitative RT-PCR of *TCL1* expression at 6 h of stimulation.

Fig. 5. TORC2 responds to BCR signaling by leaving the *TCL1* promoter and cytoplasmic translocation. (*A*) Immunoblot for TORC2 and β-actin in lysates from a mixture of naïve, GC, and memory B cells (lane 1), GC and memory B cells (lane 2), naïve B cells (lane 3), GC B cells (lane 4), and memory B cells (lane 5). (*B*) Immunoblot for TORC2 showing phosphorylated and nonphosphorylated forms with IgM stimulation of Ramos B cells for the times indicated. (*C*) Immunofluorescent staining for TORC2 in Ramos cells stimulated with IgM and/or dbcAMP at 3 h. Numbers in parentheses indicate the number of cells showing a similar staining pattern with each condition. (*D*) ChIP assay with Ramos B cells with or without 10 μg/ml anti-IgM stimulation for 1 h. PCR was performed from immunoprecipitated chromatin fragments by using anti-TORC2 or control anti-Ig Abs and primers from SI Fig. 10. (*E*) TORC2-specific immunohistochemical stain of GC B cells from human tonsil. Note the presence of an unstained macrophage (denoted by a red arrow) and the exclusive cytoplasmic localization of TORC2.

indicating that enhanced pCREB-133 does not transactivate *TCL1*. Instead, the data strongly suggest an alternative mechanism for BCR-mediated *TCL1* repression.

A critical step in the GC reaction occurs when CD40 on B cells binds its ligand, CD154, expressed on T and other cells, which may result in B cell proliferation, survival, or *Ig* class switch recombination (6). We postulate that this GC signal might, like BCR signaling, also repress *TCL1* in Ramos B cells. Consistent with this idea, anti-CD40 stimulation resulted in *TCL1* repression (Fig. 4 *D* and *E*). When Ramos cells were coincubated with anti-IgM and anti-CD40, *TCL1* repression remained at levels seen with either treatment alone, suggesting that these two signaling pathways both use a pCREB-133-independent mechanism to repress *TCL1*. Together, these data show that activation of key GC-related signaling pathways in Ramos B cells recapitulates the physiologic repression detected for *TCL1* during the GC reaction *in vivo* (12).

BCR and CD40L-Mediated TORC2 Phosphorylation and Cytoplasmic Translocation. Despite its expression in pre-B and GC-derived B cell lines (Fig. 3*A*), it was unknown whether primary B cells express TORC2. TORC2 protein expression was robust in all fractionated human tonsil subsets examined, including naïve (CD10+, IgD+), GC (CD10+, IgD−), and memory (CD10−, IgD) B cell subsets (Fig. 5*A*). TORC2 expression was comparable to the expression level detected in HEK293T cells (Fig. 2*C* and data not shown).

We postulated that the BCR- or CD40-mediated TCL1 re-

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pression in Ramos B cells was from an alteration of TORC2 expression and/or nuclear localization. No significant change in the level of TORC2 protein was detected with anti-IgM incubation, whereas TORC2 was mainly unphosphorylated (active) at rest and underwent robust phosphorylation at Ser-171, as indicated by a previously characterized mobility shift (34), with 1 h of anti-IgM stimulation, followed by a return to baseline at 6 h (Fig. 5*B*). Immunofluorescence at 3 h of anti-IgM showed that TORC2 was translocated from a mainly nuclear location almost completely into the cytoplasm in 30 of 30 cells examined (Fig. 5*C*). This translocation was transient, because at 6 h of anti-IgM TORC2 returned to the nucleus in all cells examined (data not shown). Supporting relocalization away from the *TCL1* promoter, ChIP demonstrated TORC2 at the *TCL1* promoter in unstimulated Ramos cells, whereas after 1 h of anti-IgM stimulation TORC2 was absent from the *TCL1* promoter, strongly supporting the immunofluorescence results (Fig. 5*D*). Enhanced pTORC2–171 and cytoplasmic relocalization are consistent with pCREB-133-independent, TORC2-dependent control of *TCL1* and provide a novel mechanism for *TCL1* repression *in vitro* and potentially within GCs *in vivo*. Supporting this model, robust TORC2 expression was localized in the cytoplasm of activated GC B cells in human tonsil (Fig. 5*E*).

Stimulation with cAMP results in the nuclear retention of TORC2 even in the presence of additional signals that drive TORC2 out of the nucleus (34). Because cAMP resulted in increased *TCL1* expression (Fig. 1), the localization of TORC2 was determined after preincubation with 0.1 mM cAMP followed by 3 h of anti-IgM stimulation. Consistent with results in nonlymphoid cells (34), preincubation with cAMP left TORC2 in the nucleus even with anti-IgM stimulation (Fig. 5*C*), establishing a signaling hierarchy for regulating TORC2 localization and target gene responses in B cells.

TCL1 was also repressed after anti-CD40 stimulation (Fig. 4*D*). To determine whether TORC2 cytoplasmic redistribution was also a mechanism for CD40-mediated *TCL1* repression, immunofluorescence was performed. At 3 h of anti-CD40 stimulation, TORC2 was primarily in the cytoplasm in 24 of 30 cells examined (SI Fig. 10). Anti-CD40 stimulation was also unable to translocate TORC2 out of the nucleus after preactivation with cAMP. Combined, the data strongly support the hypothesis that *TCL1* expression depends on TORC2 nuclear localization and interaction with the *TCL1* promoter and that certain GC signaling pathways repress TORC2 target genes, such as *TCL1*.

cAMP Protects B Cells from BCR and CD40L-Induced TCL1 Repression and Apoptosis. TORC2 control of *TCL1* expression predicts that *TCL1* will remain at prestimulation levels with cAMP and IgM treatments. Consistent with this prediction, treatment of Ramos B cells with IgM or CD40 resulted in decreased *TCL1* mRNA levels, whereas preincubation with cAMP inhibited *TCL1* repression (Fig. 6 *A* and *B*). At 40 h of stimulation, whereas TCL1 protein was decreased by IgM or CD40 stimulation, preincubation with cAMP blocked TCL1 down-regulation (Fig. 6*C*). These data reinforce the model for a signaling hierarchy acting on TCL1, modulated at least in part by pTORC2–171 and subcellular localization (Fig. 6).

cAMP inhibition of *TCL1* repression suggests that cAMP might protect IgM-treated B cells from death at least partially because TCL1 is a prosurvival oncoprotein with its survival effect linked to BCR signaling (18). Stimulation of Ramos cells with anti-IgM resulted in increased apoptosis, as previously shown (Fig. 6*D*) (33). Consistent with a predicted prosurvival effect, 0.1 mM dbcAMP preincubation delayed the onset and reduced the extent of apoptosis in Ramos B cells, which could be tumor-promoting within the GC over time (18). These data validate previous results for rescuing Ramos from BCR-induced
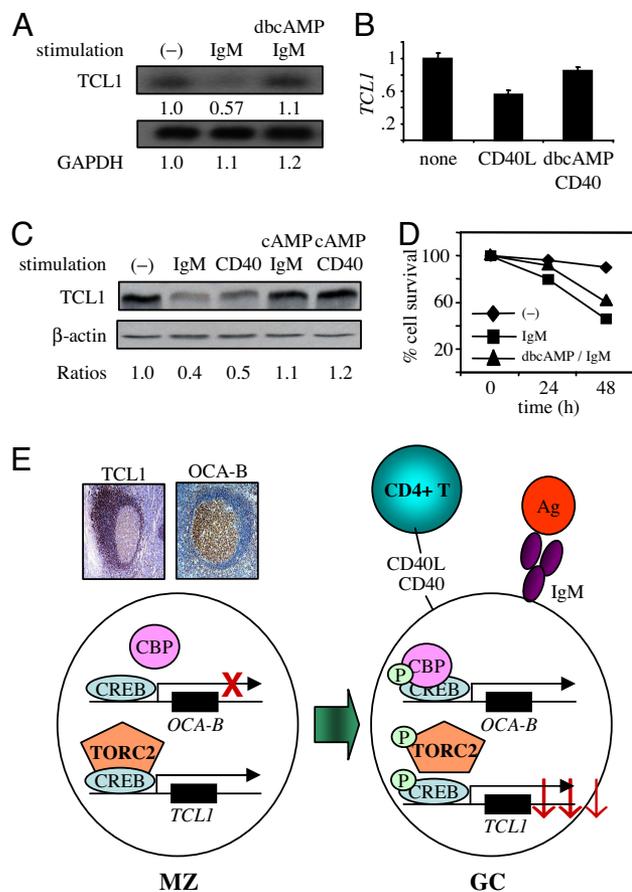
Fig. 6. BCR- and CD40-induced TCL1 repression and apoptosis are rescued by cAMP. (*A*) Northern blot for *TCL1* and *GAPDH* at 6 h of incubation with 10 μg/ml anti-IgM and/or 0.1 mM dbcAMP in Ramos B cells. (*B*) Real-time quantitative RT-PCR for *TCL1* at 6 h of incubation with 1 μg/ml anti-CD40 and/or 0.1 mM dbcAMP in Ramos B cells. (*C*) Immunoblot for TCL1 and β-actin in lysates from Ramos cells incubated at 40 h with 10 μg/ml anti-IgM, 1 μg/ml anti-CD40, and/or 0.1 mM dbcAMP. Densitometry quantification is shown in *Lower*. (*D*) Ramos cell survival measured by annexin V and propidium iodide exclusion with 10 μg/ml anti-IgM and/or 0.1 mM dbcAMP for the times indicated. (*E*) Model of CREB-TORC2 regulatory node and effects on *OCA-B* (36) and *TCL1* (12, 15, 36) expression levels in mature human B cells during the GC reaction. Immunostain panels show absent OCA-B expression in pre-GC mantle zone (MZ) B cells and high expression in GC B cells, whereas TCL1 is highly expressed in mantle zone B cells and strongly repressed in GC B cells. See *Discussion* for details.

apoptosis by cAMP with retained TCL1 expression imparting at least a component of this protection (35). These findings also provide a mechanism for enhanced survival fostering lymphomagenesis by enforced *TCL1* expression in transgenic mouse GC B cells, or in human GC B cell lymphomas that aberrantly express *TCL1*, by avoiding BCR-dependent negative selection.

Discussion

The GC B cell gene regulatory program supports the generation of B cells with a robust Ab repertoire and foils the development of potentially oncogenic B cells. This regulatory program must suppress cell survival genes, such as *TCL1*, to foster negative selection and promote apoptosis. Our work exposed a regulatory circuit in which GC B cell signaling through CD40 and the BCR activates pCREB-133-responsive genes, such as *OCA-B* and *BCL2* (36, 37), and also represses certain CREB-dependent genes, of which *TCL1* is an important example, through Ser-171 phosphorylation and nuclear exclusion of TORC2. We have

shown that *TCL1* is a direct target of the CREB-TORC2 complex and that repression depends on pTORC2–171 but not pCREB-133. Our data provide a model for constitutive expression of *TCL1* by CREB-TORC2 and Sp1 (22) in developing B cells until they enter the GC follicle center, where down-modulation provided by GC signaling phosphorylates TORC2 to drive it from the nucleus (Fig. 6*E*).

The *TCL1* reporter shows high basal activity in many different cell types including those that do not express endogenous *TCL1* (22). Our results support a mechanism for high basal promoter activity dependent on CREB levels and TORC2 levels and location. Interestingly, expression of a CREB Ser-133-Ala mutant did not reduce *TCL1* promoter function, as anticipated, but instead supported promoter activity alone or in combination with TORC2. This result indicates that genes within the GC B cell regulatory program, such as *TCL1*, may be CREB-dependent, but CBP- or p300-independent, a prediction supported by reported data showing that CBP or p300 individually is not required for peripheral B cell function or development (38).

The reduction of *TCL1* expression in Nalm-6 cells with >95% *TORC2* repression was ≈40% (Fig. 3*C*), which appears modest when compared with the decrease in *TCL1* levels after anti-IgM and CD40 stimulation (Fig. 4*E*). There are several possible explanations for this result, including that generation of stable TORC2 RNAi-expressing cells with puromycin selection could have eliminated TCL1-low-targeted cells, because *TCL1*-reduced Nalm-6 cells have a significant survival disadvantage (unpublished results). Also, *TCL1* regulators in addition to TORC2, such as Sp1, may be impacted by GC-related IgM or CD40 signaling. It is important to stress that the 40% reduction in *TCL1* actually parallels the extent of *TCL1* reduction in primary GC B cells, suggesting that TORC2 controls this component of *TCL1* gene expression.

The pCREB-133-independent circuit, mediated by the activity of the essential CREB coactivator TORC2 as shown here for *TCL1*, likely constitutes a new program of GC B cell regulation. Until this report, the only established physiologic role for TORC2 control of CREB-dependent gene expression was in cell metabolism (32, 34, 39–41). Our results provide a mechanism for constitutive CREB activation of certain genes, including *TCL1*, in the absence of cAMP stimulation in B cells (31). *TCL1* is an example of survival gene that is constitutively activated by a CREB-TORC2 complex independent of inducible CREB phosphorylation.

TORC2 dependency resolves the paradoxical effect of CREB-dependent *TCL1* repression by BCR stimulation in Ramos B cells. BCR engagement markedly increased pCREB-133, yet *TCL1* was repressed. We discovered that pTORC2–171 was translocated out of the nucleus with CD40 or BCR activation, repressing *TCL1* expression. TORC2 was previously shown to traffic in and out of the nucleus in response to metabolic signaling (32, 41). Now we report that GC-related CD40 and BCR signaling also results in pTORC2–171 translocation out of the nucleus, providing an example, along with *OCA-B*, of coactivator control of the GC B cell regulatory package (18, 42). These results suggest, like BCL6 repression of p53 to permit somatic hypermutation and class switch recombination of *Ig* genes (43), a critical role for CD40- and/or BCR-mediated pTORC2–171 emigration from the nucleus to facilitate FAS elimination of detrimental GC B cells (18) by TCL1 repression (Fig. 6*E*). Either CD40 or BCR signaling alone initiates apoptosis and negative selection in the absence of cosignaling (6, 33, 44). However, FAS-susceptible TCL1-repressed GC B cells can be rescued from death by TCL1-independent increases in the expression of BCL2L1 (Bcl-X$_L$) and CFLAR (Flip) (45–47). Aberrant TCL1 expression in TCL1-transgenic mice or by defective TORC2-mediated TCL1 repression favors GC B cell

survival independent of antigenic rescue (18) to help drive malignant transformation.

Our results suggest that pTORC2–171 is an important determinant of GC B cell fate. It will be interesting to identify additional TORC2 target genes that may also be repressed to facilitate the GC reaction. Introduction of a dominant negative TORC2 expression construct harboring a Ser-171→Ala-171 mutation into B cells with anti-IgM or anti-CD40 stimulation could further define the role of TORC2 in negative selection and B cell oncogenesis. Unfortunately, multiple attempts and strategies for overexpressing TORC2 in B cells so far have been unsuccessful because transduced cells typically underwent apoptosis (data not shown).

There already is a recognized role for hyperactive CREB-dependent gene regulation in cancer (48). CREB overexpression associates with a poor outcome in acute myeloid leukemia, and CREB transgenic mice develop a myeloproliferative disease (49, 50). Hyperactive CREB also associates with overexpression of *BCL2* in follicular lymphoma (51). Our data suggest that defects in a CREB-TORC2 regulatory node, which likely initiates *TCL1* repression in GC B cells, could be responsible for cases of GC-experienced lymphomas that maintain oncogenic *TCL1* expression.

Materials and Methods

Human Cell Lines, Tissues, and Chemicals. HEK293T, Jurkat, Nalm-6, and Ramos cell lines were maintained in RPMI medium 1640 with 10% FBS and antibiotics. Primary B-CLL were maintained as described (29). Fresh tonsil was sorted into mature B cell subsets and verified as described (12). Tonsil sections were retrieved from the pathology archives at University of California, Los Angeles. Human materials were used under institutional review board-approved protocols. dbcAMP and AICAR were from Sigma (St. Louis, MO).

Expression Vectors and Retroviruses. The −424luc *TCL1* reporter gene was previously described (22). An altered −424luc reporter was generated (QuikChange kit; Stratagene, La Jolla, CA) to disrupt the CRE-like half-site by a GACGT→GATCT mutation. *CREB*, *mCREB* (Ser-133→Ala-133), and *TORC2* expression plasmids were from M. Montminy (The Salk Institute, La Jolla, CA). A *TORC2* siRNA hairpin oligonucleotide (sequence available upon request) was cloned 3′ of an H1 promoter-modified *pQCXIP* (Clontech, Mountain View, CA) expression vector (S. Smale, University of California, Los Angeles). Retrovirus was generated in HEK293T cells grown in DMEM with 10% FBS and antibiotics by cotransfection with *pCL-AMPHO* using FuGENE 6 (Roche, Basel, Switzerland). Viral supernatant was collected 48 and 72 h after transfection, filtered, and stored at 4°C. Nalm-6 pre-B cells (1 × 10^5 per well) were incubated with 1 ml of virus supplemented with 2 μl of polybrene and centrifuged at 1,800 × g for 1 h at 30°C, which was repeated once the following day. One day after repeat infection, puromycin (1 μg/ml) was started and GFP expression was monitored. *TORC2* RNA depletion was determined by Western blot.

RNA Analysis. Total RNA was extracted by using TRIzol (Life Technologies, Carlsbad, CA). Northern blot for *TCL1* and *GAPDH* was as described (12). cDNA was made by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time quantitative RT-PCR (SYBRgreen) was performed by using an Applied Biosystems 7700 sequence detector as described (52). Expression was normalized to a *36B4* control. Primer and probe sequences are available upon request.

Protein Analysis and Abs. Western blots were as described (12), with the following modifications. Between 20 and 40 μg of

whole-cell lysate for each sample was separated by 8% SDS/PAGE and transferred to a nitrocellulose membrane. Blocked membranes were incubated with CREB (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), pCREB-133 (1:1,000; Upstate, Billerica, MA), TORC2 (1:3,000; M. Montminy), TCL1 (1:7,500), and β-actin (1:5,000; Sigma, St. Louis, MO) antiserum in TBST and 5% milk overnight. Cell stimulations were performed with anti-CD40 (K. Zhang, University of California, Los Angeles) or anti-IgM (Jackson ImmunoResearch, West Grove, PA) Abs.

Apoptosis Assay. Apoptosis was measured by annexin V binding and propidium iodide permeability (BD Pharmingen, Franklin Lakes, NJ). Flow cytometry was performed on a Coulter Elite (Beckman Coulter), and data were analyzed by using FCS Express v2.0 (DeNovo Software).

Immunofluorescence Microscopy. Ramos cells were plated on poly-lysine coverslips, washed, fixed with paraformaldehyde, washed, blocked with 0.2% BSA, incubated with anti-TORC2 Ab, washed, incubated with anti-rabbit-FITC, and washed again. Cells were counterstained with DAPI. Microscopy was performed by using a Zeiss Axioskop 2 plus microscope with a Plan-APOCHROMAT ×63/1.0 oil objective. Images were acquired by using a Zeiss Axiocam camera and Axiovision version 3.01 software.

Immunohistochemistry. Formalin-fixed paraffin-embedded sections of human tonsil were stained with TORC2 antiserum (1:1,000) by using immunoperoxidase techniques as described (12, 15).

SI. For more information on additional assays, ChIP, and transient transfection, see *SI Methods*.

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