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BCL6 Controls the Expression of the B7-1/CD80 Costimulatory Receptor in Germinal Center B Cells

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Abstract

The BCL6 proto-oncogene encodes a transcriptional repressor required for the development of germinal centers (GCs) and implicated in the pathogenesis of GC-derived B cell lymphoma. Understanding the precise role of BCL6 in normal GC formation and in lymphomagenesis depends on the identification of genes that are direct targets of its transcriptional repression. Here we report that BCL6 directly controls the expression of B7–1/CD80, a costimulatory receptor involved in B–T cell interactions critical for the development of T cell–mediated antibody responses. Upon CD40 signaling, transcription of the CD80 gene is induced by the nuclear factor (NF)-κB transcription factor. Our results show that BCL6 prevents CD40-induced expression of CD80 by binding its promoter region in vivo and suppressing its transcriptional activation by NF-κB. Consistent with a physiologic role for BCL6 in suppressing CD80, the expression of these two genes is mutually exclusive in B cells, and BCL6-defective mice show increased expression of CD80 in B cells. The results suggest that BCL6 may directly control the ability of B cell to interact with T cells during normal GC development. In addition, these findings imply that T–B cell interactions may be disrupted in B cell lymphoma expressing deregulated BCL6 genes.

Key words: BCL6 • germinal center • CD80 • CD40 • lymphoma

Introduction

The BCL6 proto-oncogene was identified by virtue of its involvement in chromosomal translocations associated with B cell non-Hodgkin lymphoma (NHL),* including diffuse large cell lymphoma (DLCL) and, more rarely, follicular lymphoma (1–6). The product of the BCL6 gene is a nuclear phosphoprotein belonging to the POZ/Zinc finger (ZF) family of transcription factors (7). It can bind DNA specifically via its six ZFs and functions as a potent transcriptional repressor by recruiting a number of corepressor molecules (8–12). In the B cell lineage, the BCL6 protein is expressed by the mature B cells within germinal centers (GCs), but not by naive pre-GC B cells or by post-GC memory and plasma cells (13–15). BCL6 serves a critical function in the regulation of T cell–mediated antibody responses, as mice lacking BCL6 cannot form GCs and, accordingly, fail to mount a secondary T cell–dependent response due to lack of antibody affinity maturation (16, 17).

Consistent with its essential role in GC formation, the signals that regulate BCL6 expression are also critical for GC development. At the protein level, B cell receptor (BCR) engagement by antigen induces MAP kinase–mediated phosphorylation of BCL6, which, in turn, targets BCL6 for degradation by the ubiquitin proteasome pathway (18). At the transcriptional level, a variety of stimuli, including BCR activation, CD40 receptor engagement, and mitogenic stimulation, appear to down-regulate BCL6 RNA expression in spleen- and peripheral blood–derived B cells (14), although the effect of these signals has not been verified in GC B cells. Normal transcriptional regulation of the BCL6 gene is disrupted by tumor-associated chromosomal translocations which juxtapose heterologous promoters to the coding exons of BCL6, causing its deregulated expression by a mechanism called promoter substitution (19). The 5′ noncoding region of the BCL6 gene is also subjected to somatic hypermutation in normal GC B cells as well as in GC-derived NHL (20–23). While the role of these mutations in normal B cells is unclear, preliminary evidence suggests that some tumor-associated

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*Abbreviations used in this paper: BCR, B cell receptor; ChIP, chromatin immunoprecipitation; GC, germinal center; GFP, green fluorescent protein; NF, nuclear factor; NHL, non-Hodgkin lymphoma.

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mutations may deregulate BCL6 transcription (24; unpublished data).

The function of BCL6 in GC formation presumably depends upon its ability to repress the transcription of specific target genes. BCL6 can bind to the same DNA sequence recognized by the STAT6 transcriptional activator, the main nuclear effector of IL-4–induced signaling (16, 25). In this way, BCL6 can repress the STAT6-mediated, IL-4–induced expression of Ig germ line ε transcripts, therefore modulating the isotype switch toward IgE in vitro and in vivo (25). In addition, a variety of putative BCL6 target genes involved in the control of lymphocyte activation, differentiation, cell cycle, apoptosis, and inflammation have been proposed based on the analysis of B cells that differentially express BCL6 (26–28). However, these approaches could not distinguish genes that are direct targets of BCL6-mediated transcriptional suppression from those whose response is secondary to the biological changes induced by BCL6. Consequently, the precise function of BCL6 in GC development and lymphomagenesis remains obscure.

In this study we have identified a gene whose transcription is directly regulated by BCL6. The results show that BCL6 binds to the promoter region and modulates the transcription of the CD80/B7–1 (CD80) gene, which encodes an important costimulatory molecule involved in B–T cell interactions. CD80 and its related molecule CD86 (B7–2) are expressed by antigen-presenting cells including B cells, and their interaction with the CD28 and CTLA-4 cognate molecules is required for T cell activation, GC formation, Ig class switching, for the rejection of grafts and for antitumor immunity (29–31). CD80 is expressed at extremely low levels in naive B cells and in normal and neoplastic GC B cells, while its expression is high in memory B cells and can be induced by CD40 signaling on naive B cell in vitro (32, 33). These observations suggest that the regulation of CD80 expression is tightly controlled during B cell development and function, and that this regulation is critical for the development of T cell–mediated antibody responses. Our findings indicate that the BCL6 protooncogene has a direct role in controlling CD80 during GC development and that CD-40–mediated down-regulation is necessary for CD80 induction. These results have also implications for the pathogenesis of lymphomas in which BCL6 expression is deregulated.

Materials and Methods

Cell Lines. All B cell lines were cultured in IMDM supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine. The Phoenix viral packaging line was grown in DMEM, 10% FCS, 300 μg/ml Hygromycin, 1 μg/ml Diphtheria Toxin. The 293T fibroblast cell line expressing CD40L or CD8 was maintained in DMEM with 10% FCS and 300 μg/ml Hygromycin. NTera-2 cells were cultured in DMEM containing 10% FCS, penicillin/streptomycin and 2 mM L-glutamine.

Retroviral Infection and FACS® Sorting. PINCO-HA-BCL6 and PINCO-HA-BCL6$_{\Delta PEST}$ were constructed by blunt-end ligation of HA-BCL6 or HA-BCL6$_{\Delta PEST}$ sequences (18) into the BamHI site (filled-in) of the PINCO retroviral expression vector (34). The amphotropic packaging cell line Phoenix was transfected with PINCO, PINCO-HA-BCL6, and PINCO-HA-BCL6$_{\Delta PEST}$ by the calcium-phosphate/chloroquine method as described in http://www.stanford.edu/group/nolan/NL-phnxr.html, and viral supernatants were collected 48 h after transfection. For infection, Ramos B cells (5×10^4 /ml) were resuspended in the viral supernatant supplemented with 0.5 μg/ml polybrene, centrifuged for 45 min at 1,800 rpm, and incubated at 37°C for 2 h. Four cycles of infection were performed before cells were placed back in growth medium. Green fluorescent protein (GFP) expression in Phoenix (~100%) and Ramos cells (10–30%) was analyzed by fluocytometry using a FACSCalibur™ (Becton Dickinson); transduced GFP$^+$ Ramos cells were selected by cell sorting using a FACStar™ (Becton Dickinson). Clones were isolated and characterized by Western blot analysis for HA-BCL6 and HA-BCL6$_{\Delta PEST}$ expression using anti-BCL6 (N3; Santa Cruz Biotechnology, Inc.) and anti-HA antibodies (clone 12CA5; Boehringer).

CD40 Stimulation. Induction of CD40 signaling in the B cell lines was performed either by treatment with agonistic anti-CD40 antibody (G28–5 hybridoma from American Type Culture Collection; 0.5 μg/ml) or by cocultivation with 293T fibroblast monolayers engineered to express the CD40 ligand (CD40L) or CD8 (as negative control). For cocultivation, B cells and 293T cells were mixed at 2:1 ratio in 6 well plates, centrifuged at 1,000 rpm for 5 min, and incubated at 37°C for the indicated interval before harvesting.

Flow Cytometric Analysis. Ramos cell clones (PINCO, PINCO-HA-BCL6, and PINCO-HA-BCL6$_{\Delta PEST}$) stimulated with CD40 signaling by cocultivation with 293T (CD40L) fibroblasts were analyzed on a FACSCalibur™ (Becton Dickinson) for surface molecule expression using PE-conjugated anti-human B7.1 (CD80), B7.2 (CD86), FAS (CD95), CD23, and CD54 antibodies (BD Biosciences).

Northern and Western Blot Analysis. Total RNA was isolated using the Trizol-reagent (GIBCO BRL) and Northern blot analysis was performed by standard methods with P^{32}-labeled probes for BCL6 and β-actin. The protocol for Western blot analysis of BCL6 expression has been described (18).

Quantitative RT-PCR Analysis. 2 μg of total RNA were reverse transcribed into cDNA by using the First Strand cDNA Synthesis Kit (Invitrogen). Quantitative RT-PCR was performed with the DNA Master SYBR Green I kit reagents in the LightCycler instrument (Roche), and the LightCycler Software 3 (Roche) was used for data analysis. The oligonucleotide sequences used are as follows. For CD80 (CD80): 5′-CAC CTG GCT GAA GTG AC-3′ and 5′-GTC AGG CAG CAT ATC AC-3′; for FAS: 5′-CAA ATG CAG AAG ATG TAG-3′ and 5′-GAT TCA TGA GAA CCT TGG-3′; for CD38: 5′-GCC CAG ACT GGA GAA AGG AC-3′ and 5′-CAA CCA CAG CGA CTG GCT CA-3′.

Transient Transfection and Reporter Gene Assays. The CD80-TK-CAT reporter construct and its linker-scanning mutants (M1-M18) were kindly provided by L. Glimcher, Harvard University, Boston, MA (35). Transient transfections in NTera-2 cells were performed by calcium-phosphate precipitation using 2 μg of the reporter plasmid, various amounts of the BCL6 expression vector pMT2T-HA-BCL6, and the nuclear factor (NF)-κB c-Rel expression vector (p-MT2T-c-Rel), as indicated in the figure legend. The Renilla luciferase pRLTK vector (0.3 μg) was used as internal control for transfection efficiency. CAT activities were measured after 48 h using the CAT Enzyme Assay System from

Promega, while luciferase activity was measured using the Dual-Luciferase Reporter Assay kit (Promega). The expression levels of BCL6 and its mutant were controlled by Western blot analysis using anti-HA antibodies.

Chromatin Immunoprecipitation Assay. For chromatin immunoprecipitation (ChIP), Ramos cells ($\sim 10^8$ total), or transiently transfected NTera-2 cells (one 10 cm plate per assay, harvested 48 h after transfection) were incubated with formaldehyde (1% final volume) for 10 min at 37°C. Cells were washed in PBS containing protease inhibitors, resuspended in 200 μl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.0), and incubated on ice for 10 min. After sonication to shear the chromatin (10–20 s for three times at 30% maximum power on ice), cellular debris was pelleted by 10 min centrifugation at 14,000 rpm. Supernatants were recovered and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, protease inhibitors [Boehringer Mannheim]); 50 μl of this solution were removed to serve as DNA input control. The diluted supernatants were precleared with 50 μl Protein G-Sepharose beads (50% Slurry) and 20 μg sonicated salmon sperm DNA for 1–2 h at 4°C with rotation. After centrifugation to remove the beads, 2 μg of the respective antibody (anti-BCL6 [N3], anti-c-Rel [C], anti-p50 [NLS]; Santa Cruz Biotechnology, Inc.) were added for 2 h, followed by overnight incubation with 30 μl protein G-Sepharose at 4°C with rotation. In each experiment, one sample with no Ab was included as negative control for nonspecific binding. Pelleted beads were washed sequentially with 1 ml each of the following buffers for 5 min: (a) 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, with 150 mM NaCl; (b) 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl; (c) 0.25M LiCl, 1% NP-40, 1% NaDOC, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0; (d and e) TE pH 8.0. Immune complexes were eluted off the beads by adding 250 μl 1% SDS in 0.1 M $NaHCO_3$ elution buffer and rotating the sample for 15 min at room temperature twice. To reverse the cross-links, 5 M NaCl was added to the eluates, followed by overnight incubation at 65°C and Proteinase K treatment (20 μg for 1 h at 45°C). DNA was purified by phenol-chloroform extraction, ethanol precipitated and resuspended in 30–50 μl of TE (200 μl for the total input sample). PCR reactions were performed using 2 μl of the above DNA preparations as template and the following conditions: 95°C for 2' and 25–30 cycles at 95°C for 30", 58°C for 30", 72°C for 1'. The oligonucleotide sequences were as follows: for the transfected TK-CAT and CD80-TK-CAT reporter genes: T3 primer (Promega) and 5′-GTC ACC TTA ATA TGC GAA GT-3′. For the CD80 promoter region (GenBank/EMBL/DDBJ accession no: XM_002948), three different primer pairs were used: (a) 5′-CCT TAT AGA CAT GAA CAG-3′ and 5′-CAC GGT GCT AGC CTG ACT-3′ for generation of fragment A, containing the M7 sequence (−3128 to –2946 from the transcription initiation start; reference 35); (b) 5′-CAA GAT TGC ACC ACT GCA CT-3′ and 5′-GGG AAC ATA GAT TGG AGG TA-3′ for fragment B (−1281 to −1054; reference 35); (c) 5′-CAA AGG AAC CCT TAG GGT CT-3′ and 5′-GAG CTC AAG CGA TTT TCC CA-3′ for fragment C (+296 to +485). PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining and/or by Southern blot hybridization using the P^{32}-labeled PCR product as a probe.

Analysis of BCL6-deficient Mice. Three BCL6-deficient (Δ/Δ) mice and three heterozygous littermates controls (Δ/+) were immunized intraperitoneally with sheep red blood cells and killed 6 d later. Cell suspensions from spleen were prepared as previously described, stained with appropriate combinations of fluorochrome and/or biotin-labeled monoclonal antibodies and analyzed on a FACStar$^{Plus™}$ flow cytometer (Becton Dickinson). The antibodies used were APC-anti-B220, FITC-anti-IgD, PE-anti-CD80, PE-anti-CD23, and PE-anti-CD95 (BD Biosciences).

Results

Constitutive Expression of BCL6 Inhibits CD40-induced Activation of CD80 Gene Expression. The observation that CD40 down-regulates BCL6 expression (14; unpublished data) raised the possibility that BCL6 may influence the expression of genes induced by CD40. To test this hypothesis, we infected Ramos cells, which represent transformed GC B cells, with a retroviral vector (PINCO; reference 34) that encodes HA-tagged BCL6 to obtain cells that constitutively express exogenous BCL6 since the retroviral promoter is resistant to CD40 induced down-regulation. The PINCO vector also encodes a GFP for efficient selection of transduced cells by FACS®. Ramos cells were infected with vectors (Fig. 1 a) expressing either the wild-type BCL6 protein (PINCO -HA-BCL6) or a deletion mutant (PINCO-HA-$BCL6_{\Delta PEST}$) that is still capable of transcriptional repression, but has a longer half life due to deletion of the PEST domains recognized by the ubiquitin-proteasome complex (18). Northern blot analysis of representative clones confirmed that endogenous BCL6 RNAs were down-regulated by anti-CD40 treatment in all transduced clones, while exogenous HA-BCL6 or HA-$BCL6_{\Delta PEST}$ RNAs were constitutively expressed (Fig. 1 b). Western blot analysis of the same cells showed that the BCL6 proteins were constitutively expressed in the BCL-transduced cells.

We then examined the effect of constitutive BCL6 expression on the up-regulation of several known CD40 target genes (CD80, CD86, CD95, CD11a/18, CD54, and CD23; references 36 and 37) by FACS® analysis of transduced Ramos cells cocultivated with 293T cells expressing CD40L. Fig. 2 a shows that CD40-induced up-regulation of CD80 (mean fluorescence intensity), but not of CD95, was significantly (>50%) inhibited in multiple clones of PINCO-HA-BCL6- and PINCO-HA-$BCL6_{\Delta PEST}$-transduced Ramos cells. Other CD40 target genes (CD86, CD54, CD11a/18, and CD23) were not significantly affected by BCL6 expression (data not depicted). A quantitative RT-PCR analysis of CD80 and CD95 transcripts also revealed that CD40-induced up-regulation of CD80, but not CD95, was significantly reduced cells constitutively expressing wild-type BCL6 and abrogated in those expressing $BCL6_{\Delta PEST}$ (Fig. 2 b). These results suggest that BCL6 inhibits CD40-induced expression of the CD80 gene.

BCL6 Represses NF-κB–induced CD80 Gene Transcription. As CD40 signaling up-regulates expression of its target genes primarily by activation of the NF-κB transcription factor complex (38, 39), we examined whether the BCL6 transcriptional repressor can influence NF-κB–induced transcription. For this purpose, we used transient transfection/reporter gene assays in Ntera-2 cells (which lack en-

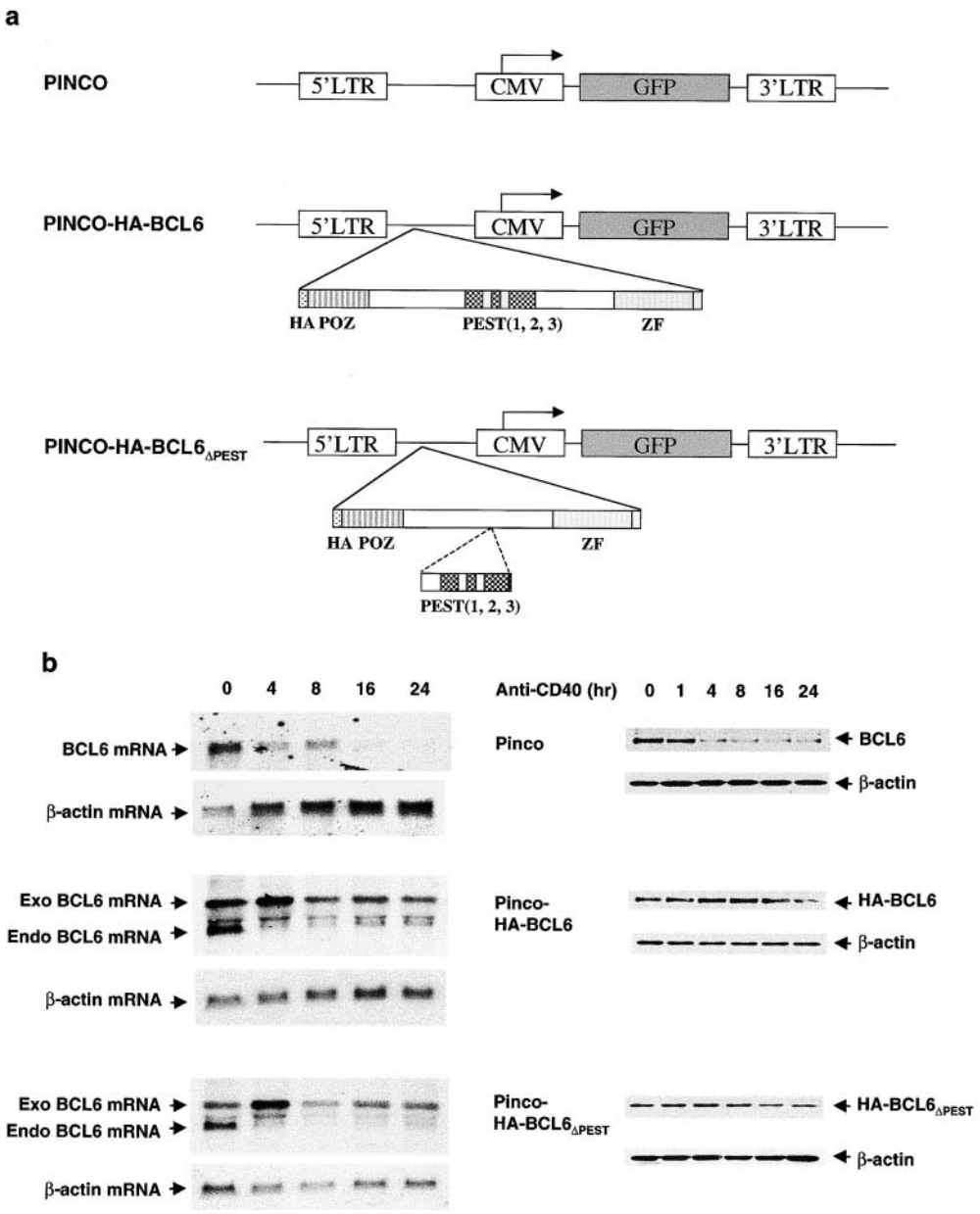


Figure 1. Constitutive expression of exogenous BCL6 in Ramos cells transduced with PINCO retroviral vectors. (a) Schematic representation of PINCO, PINCO-HA-BCL6, and PINCO-HA-BCL6Δ_{PEST} viral vectors. (b) Northern (left panels) and Western (right panels) blot analysis of endogenous (endo) and exogenous (exo) BCL6 expression in Ramos cell clones transduced with PINCO, PINCO-HA-BCL6, and PINCO-HA-BCL6Δ_{PEST} after treatment with CD40L for the indicated time. The β-actin expression was analyzed as a control.

dogenous NF-kB activity) to test whether BCL6 expression vector could modulate NF-κB–dependent (c-Rel) transcriptional activation of a CAT reporter gene linked to the 5′ enhancer region of the CD80 gene. This region was previously shown to support NF-κB-dependent CD80 gene expression in vivo (35). Fig. 3 a shows that BCL6 strongly inhibits c-Rel-induced transcriptional activation of CD80. This activity represents genuine transcriptional repression because it is significantly reduced with BCL6 deletion mutants lacking either the DNA binding (BCL6ΔZF) or the transcriptional repression (BCL6ZF) domain (8). In an analogous assay, BCL6 was also able to repress NF-κB p65–mediated transcriptional activation of the same reporter gene, as well as a reporter gene containing the B6BS BCL6-binding site (8) linked to a κB site (unpublished data). Together, these results indicate that BCL6 can modulate transcription driven by NF-κB response elements within experimental as well as native (CD80) regulatory sequences.

To localize the BCL6 response element(s) within the CD80 enhancer region, we screened a series of linker-scanning mutants of the CD80-TK-CAT reporter construct (M1-M18; reference 35) for mutants that retained NF-κB–induced activation, but displayed reduced BCL6-mediated suppression. The M9, M10, M16, and M17 mutants were insensitive to activation (Fig. 3 b) and no longer bound NF-κB in vitro (reference 35, and unpublished results). However, the M7 mutant, and in part the m15 mutant, retained NF-κB inducibility, but were significantly resistant to BCL6-mediated suppression. Notably, the M7 mutation affected a DNA site (ggtcaTCTTAGAA) closely resembling the consensus sequence previously identified for BCL6 binding in vitro (TTCYTNGAA; references 8 and

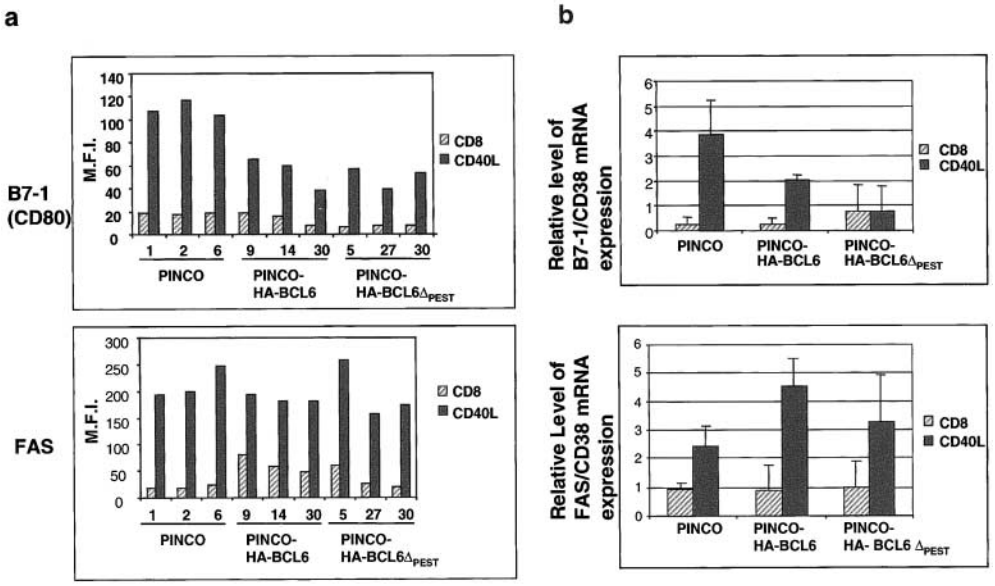


Figure 2. Constitutive BCL6 expression in Ramos cells inhibits the CD40L-induced expression of CD80 (CD80), but not of CD95. (a) FACS® analysis of CD40L-induced gene activation (CD80, CD95) in representative Ramos cell clones transduced with PINCO, PINCO-HA-BCL6, or PINCO-HA-BCL6Δ_{PEST}. (b) Quantitative RT-PCR analysis of CD40L-induced CD80 and CD95 mRNA expression in Ramos cells transduced with the indicated PINCO vectors. CD38 mRNA was used for normalization.

9), while the M15 region contains a slightly degenerated BCL6 binding site (see below). These results indicate that BCL6 can repress NF-κB–induced transcriptional activation of the CD80 enhancer, and that this repression is mediated by sequences distinct from those involved in NF-κB–mediated activation.

BCL6 Binds the CD80 Enhancer Region In Vivo. To examine whether BCL6 binds the CD80 enhancer in vivo, we performed ChIP assays in Ntera-2 cells cotransfected with vectors expressing c-Rel, p50, BCL6, a control reporter lacking CD80 sequences (TK-CAT) and a reporter gene containing either the wild-type (CD80-TK-CAT) or the M7 mutant (CD80[M7]-TK-CAT) of the CD80 enhancer. Specific binding of BCL6 to the test (region B in the CD80 containing plasmids) versus the control region (region A in TK-CAT) was assessed in the same transfected cells by using the same PCR primers (see Fig. 4 legend). Fig. 4 a shows that c-Rel and p50 can bind specifically to the wild-type CD80 enhancer and that their binding is conserved in the M7 mutant. Conversely, BCL6 can also bind to the wild-type CD80 enhancer, but not to the M7 mutant, consistent with the functional data shown in Fig. 3 b. These results indicate that BCL6 can bind the CD80 enhancer region in vivo and identify the M7 motif as the region of BCL6 binding, which is clearly distinct from that bound by NF-κB factors.

To conclusively demonstrate the physiologic nature of BCL6 binding to CD80 sequences, we examined whether endogenous BCL6 binds the native CD80 enhancer region in vivo in nontransfected Ramos cells. We analyzed by ChIP the binding of BCL6 to the enhancer region containing the sequence identified by the M7 mutant (A: −3128 to −2946) or, as controls, to two other regions of the CD80 gene (B: −1281 to −1054; C: +296 to +485; Fig. 4 b). The results show that BCL6 can bind specifically to region A, but not to the control regions B and C. Analogous experiments showed a weak binding to the m15 region that contains a degenerated BCL6 binding site (not shown). These results demonstrate that the CD80 gene is a direct and physiologic target for BCL6 binding and transcriptional repression in vivo.

Relationship between BCL6 Expression and CD80 Expression in Lymphoid Cells. The observation that BCL6 controls CD80 expression implies that the expression of these two molecules may be inversely related in B cells. Consistent with this notion, it has already been established that normal and neoplastic GC B cells and GC NHL cell lines, which all express BCL6 (13), express extremely low levels of CD80 (reference 32; and see Discussion). To examine this relationship more precisely, we examined BCL6 and CD80 expression in three pairs of Burkitt's lymphoma lines (KEM, MUTU, ODHI), each derived from the same tumor case, but selected in vitro to represent either a GC phenotype (type I) expressing BCL6, or a post-GC immunoblastic phenotype (type III) in which BCL6 expression is down-regulated (40, 41). RT-PCR analysis shows that BCL6 and CD80 RNA expression are mutually exclusive, and that CD80 RNA and protein are detectable only in type III cells that lack BCL6 (Fig. 5).

Next, we used flow cytometric analysis to compare CD80 expression in the spleens of mice lacking BCL6 (BCL6Δ/Δ) and their phenotypically normal heterozygous controls (BCL6+/Δ) (17). In BCL6Δ/Δ mice, a direct comparison between purified similar cells differentially expressing BCL6 was not possible since these mice lack GC. Therefore, CD80 expression had to be compared between whole $B220^+$ B cell populations. In wild-type mice, most $B220^+$ B cells do not express or express very low levels of BCL6, but include 5–10% GC B cells expressing high levels of BCL6 protein; conversely, in BCL6Δ/Δ BCL6 protein whose levels are increased by its longer half-life and inability to negatively autoregulate BCL6 gene transcription (17, 42, 43). Flow cytometric analysis revealed that, even within whole B cell populations, a significantly higher expression

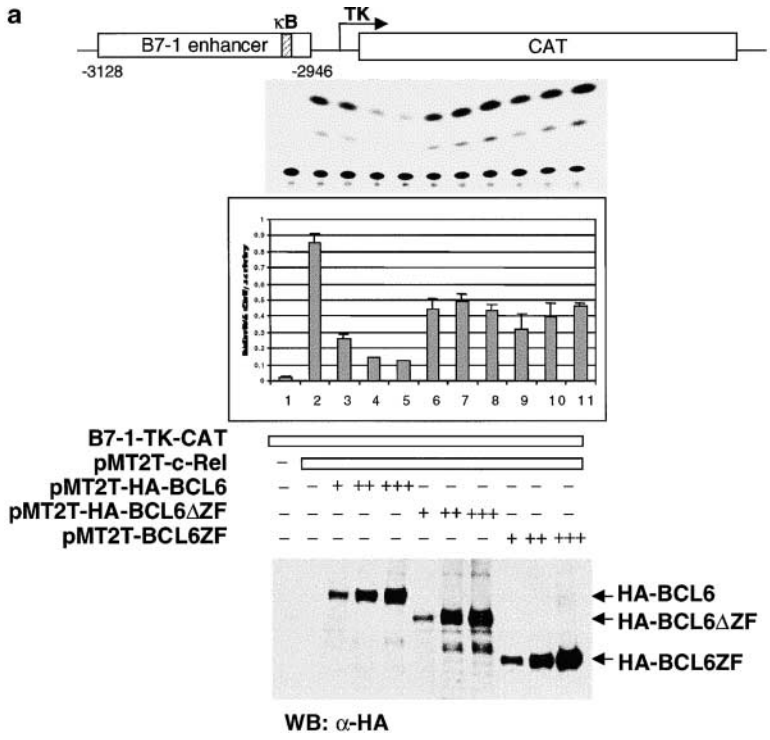
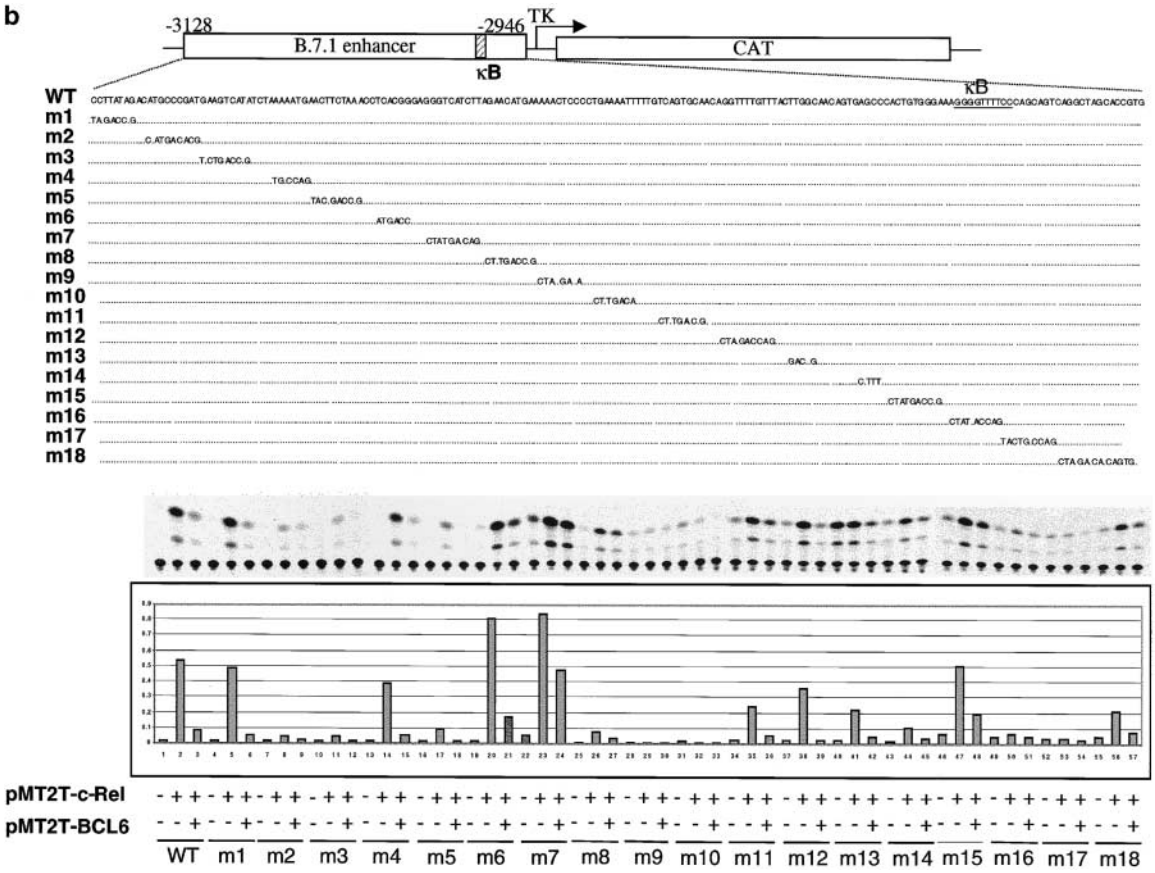


Figure 3. BCL6 represses CD40L-induced activation of the CD80 gene by inhibiting NF-κB. (a) BCL6 represses transcription of an NF-κB activated CD80 promoter-driven reporter gene. NTera 2 cells were cotransfected with 2μg of the CD80-TK-CAT reporter plasmid (on top) and increasing amount (2, 4, and 6 μg) of pMT2T-HA-BCL6, pMT2T-HA-BCL6ΔZF, and pMT2T-HA-BCL6ZF, in the absence or presence of NF-κB (c-Rel; 2 μg). CAT activities were analyzed as described in Materials and Methods. The expression levels of BCL6 and its mutant were determined by WB analysis with anti-HA antibodies (bottom panel). (b) Mapping the site responsible for BCL6 repression of the CD80 promoter. A series of linker-scanning mutants of the CD80-TK-CAT reporter plasmid (M1-M18) were cotransfected with or without 2 μg of NF-κB (c-Rel) in the absence or presence of BCL6 (4 μg) as indicated and the CAT activities were measured as compared with the wild-type reporter construct.



of CD80 expression was detectable in the splenic B cells of BCL6Δ/Δ mice both in terms of number of CD80 positive cells (Fig. 6 a: representative analysis of one mouse) and mean levels of expression per cell (Fig. 6 b: summary of results for three mice pairs). Although BCL6 Δ/Δ mice display an inflammatory disease of the spleen (17) that may also

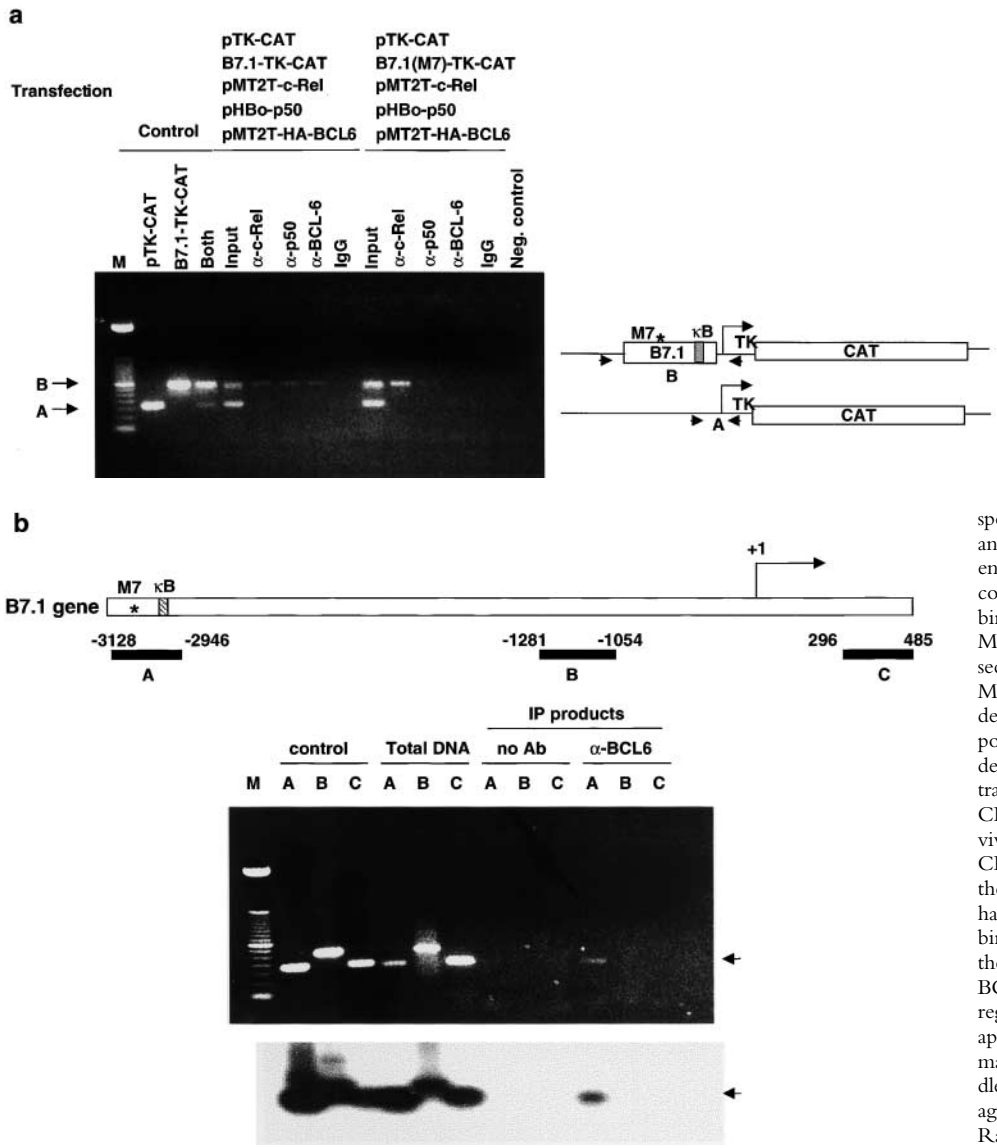


Figure 4. BCL6 binds to the CD80 enhancer in vivo in a region distinct from the NF-κB factors binding site. (a) NTera-2 cells, cotransfected with the indicated plasmids, were used to prepare chromatin and immunoprecipitations were performed with anti-c-Rel, anti-p50, anti-BCL6, or control mouse IgG antibodies as in Materials and Methods. A schematic map of the TK-CAT and CD80-TK-CAT (wild-type or M7 mutant) reporter constructs is shown on the right. The same oligonucleotides, approximately positioned below the maps (arrow), were used for amplification of the ChIP DNA, with A and B designating the respective PCR products. While c-Rel and p50 bind specifically to the CD80 enhancer region and their binding is conserved in the M7 mutant, BCL6 can bind to the wild-type, but not to the M7 mutant region, identifying the M7 sequence as the site of BCL6 binding. M: molecular weight marker (50 bp ladders). Control: plasmid DNA, used as positive control for PCR. Input: DNA derived from 10% of the chromatin extract before IP. (b) BCL6 binds to the CD80 promoter in Ramos B cells in vivo. On top, schematic of the human CD80 regulatory region; arrow indicates the transcriptional start site and the hatched box represents the NF-κB binding sequence. The asterisk denotes the M7 mutant sequence, to which BCL6 binds. The three CD80 genomic regions amplified for analysis (A–C) are approximately positioned below the map (see Materials and Methods). Middle panel: ethidium bromide stained agarose gel of PCR products A–C from Ramos cells. Chromatin was immunoprecipitated using the anti-BCL6 Ab (N3) and PCR reactions were performed on both 10% input chromatin (total DNA) and IP products. Genomic DNA was used as a positive control for the PCR (Control). The same gel was blotted and hybridized to a probe consisting of PCR-generated fragments A, B, and C (see Materials and Methods), labeled individually and added together to the hybridization mix (bottom panel).

contribute to increased CD80 expression, the results are consistent with a role for BCL6 in modulating CD80 expression in vivo. Taken together, these observations confirm that BCL6 and CD80 expression are inversely related in a variety of B cell types, consistent with the notion that BCL6 modulates CD80 expression in vivo.

Discussion

Several recent reports have identified genes whose expression is down-regulated in association with BCL6 expression and that have roles in B cell activation, differentiation, inflammation, cell cycle control, and apoptosis (26–28). Nonetheless, these genes could represent either direct targets of BCL6 or indirect targets whose expression is down-regulated in response to the changes induced by BCL6 in the particular cell type used in the experiment. The identification of direct targets is important in distinguishing the precise function of a transcription factor independently of the "downstream" changes induced in the cell. The results herein, and in particular the chromatin immunoprecipitation experiment shown in Figs. 3 and 4, show that BCL6 binds directly to the CD80 promoter region in vivo, and thus demonstrate that down-regulation of CD80 expression is part of the specific activity of BCL6 in B cells. These results also indicate that BCL6 can bind in vivo to a DNA site (ggtcaTCTTAGAA) slightly different from the consensus sequence previously identified for BCL6 binding in vitro (TTCYTNGAA) (8, 9). This newly identified BCL6

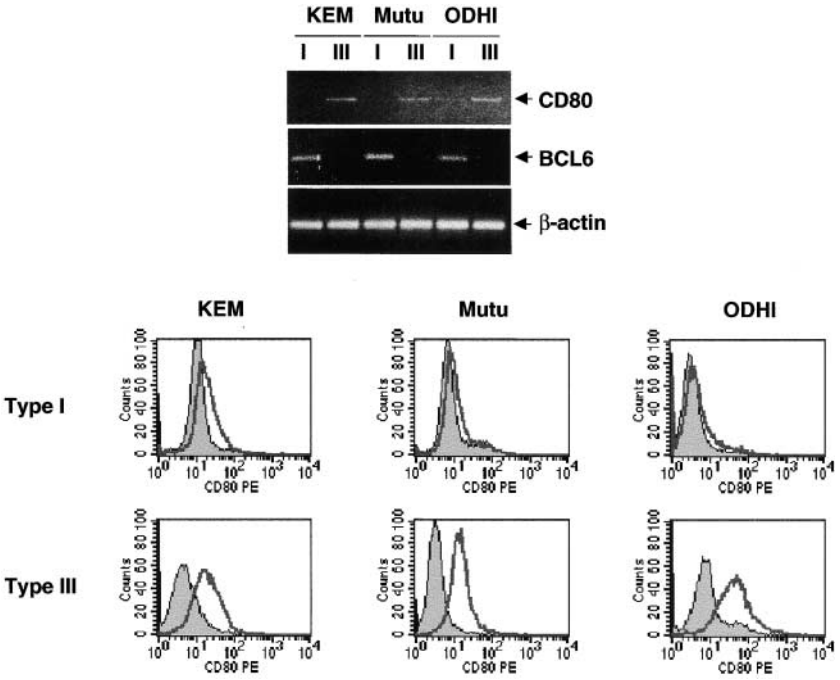


Figure 5. Inverse expression pattern of BCL6 and CD80/CD80 in Burkitt's lymphoma cell lines. RT-PCR (top panel) and FACS® (bottom panel) analysis of CD80/CD80 expression in different Burkitt's lymphoma lines (type I and type III). The shaded areas represent the background binding of control (isotype- and species-matched) antibodies (which vary among the various cell lines as expected), while the areas delimited by the green lines indicate show the reactivity of the anti-CD80 antibodies.

binding motif may be useful for reevaluating a number of other candidate BCL6 target genes.

These findings also suggest that BCL6 has a strong inhibitory effect on NF-κB–induced transcription. Notably, BCL6 and NF-κB complexes recognize different DNA motifs (44), and BCL6 does not prevent DNA binding by NF-kB in vitro or in vivo (Fig. 4, and unpublished data). Furthermore, recent results indicate that, consistent with a role of CD40 in down-regulating BCL6, NF-kB and BCL6 are not active at the same time during GC development (unpublished data; see below). Together these observations suggest that these proteins may exert opposing effects by sequentially recruiting transcriptional repressors (BCL6) or activators (NF-κB) to the same promoters at different stages of GC transit. Thus, the role of BCL6 in normal GC B cells may be to prevent premature or subliminal activation of NF-κB–dependent transcription before optimal CD40 signaling ensues.

These results show that BCL6 may control some (e.g., CD80 transcription), but not all (e.g., CD95 transcription) aspects of the NF-κB–mediated CD40 response. This finding can be explained by the observation that CD40-mediated activation of CD80 is exclusively dependent on NF-κB activation, while the activation of other CD40-induced (and "BCL6-insensitive") genes is partially (CD95, CD23, and CD54) or completely (CD11a/18) independent of NF-κB activation (45). Thus, the activity of BCL6 may be predominantly directed to control those genes that are induced by CD40 via NF-κB.

Several observations suggest that the negative control of CD80 expression by BCL6, which was detected here under experimental conditions, has in fact physiological significance. The most direct evidence for this notion would be the demonstration that CD80 is not expressed in normal human GC B cells and GC-derived lymphoma expressing

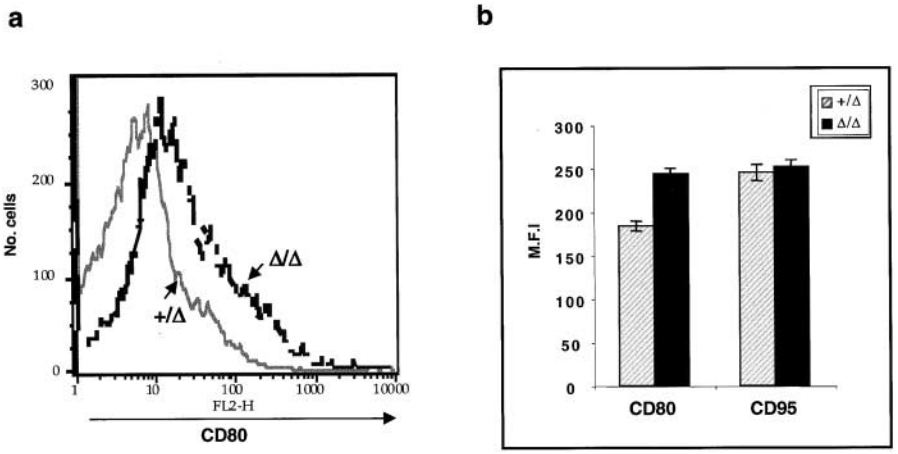


Figure 6. CD80 (CD80) expression in BCL6 deficient mice. (a) FACS® analysis of CD80 (CD80) expression in B220$^+$ splenic B cell populations from a representative BCL6 Δ/Δ mouse and a control heterozygous (BCL6 Δ/+) mouse. (b) Expression of CD80 and CD95 (control) in splenic B-cells (B220$^+$) obtained by FACS® analysis of three BCL6 Δ/Δ mice versus three littermate controls. Results are expressed as mean fluorescence intensity, with bars indicating standard deviations.

BCL6. Unfortunately, there has been considerable controversy regarding the level of expression of CD80 in normal and neoplastic mature B cells due to the poor performance of available antibodies in immunohistochemical analysis of tissue sections (discussed in reference 32; and our unpublished data). However, by comparing different antibodies and methods, it has been ascertained that both normal GC B cells and GC-derived NHL, both expressing BCL6, have extremely low levels of CD80 (32, 33). Consistent with this notion, our results confirm that BCL6 and CD80 expression are mutually exclusive in GC-derived B cell lines, and indicate that CD80 expression is up-regulated in BCL6-deficient mice (Figs. 5 and 6). Furthermore, information on CD80 expression within the GC can be indirectly derived from the observation of the distribution of nuclear NF-κB, the transcriptional mediator of CD40 induced CD80 expression. Toward this end, recent studies showed that most GC B cells express BCL6 and inactive cytoplasmic NF-κB complexes, while active nuclear NF-kB is detectable only in a small fraction of GC centrocytes which, significantly, do not express BCL6 (unpublished data). These observations indicate that BCL6 may control CD80 expression in GC centroblasts and most centrocytes, while T cell–induced CD40 signaling may down-regulate BCL6 hence allowing CD80 expression in late centrocytes.

The finding that BCL6 controls CD80 gene expression has implications for the role of BCL6 in normal GC development. The interaction of CD80 with the CD28 molecule expressed by T cells is required for the acquisition of helper T cell functions, and is critical for the development of T cell mediated antibody responses in GC (29, 46). By controlling CD80 expression in GC centroblasts, BCL6 may prevent a full interaction of B cells with helper T cells before the GC has expanded sufficiently and somatic hypermutation is complete. Interestingly, analogous to CD40, BCR signaling also induces CD80 (47) and represses BCL6 expression (18). Thus, it appears that multiple signaling pathways controlling CD80 expression must first be modulated (by BCL6) and then released (by down-regulating BCL6) during GC transit and selection of B cells.

Finally, these results have implications for the role of BCL6 in lymphomagenesis. A sizable fraction of diffuse large cell lymphomas (>40%) carry translocations that deregulate BCL6 expression by promoter substitution (1, 19, 48). The heterologous promoters juxtaposed to the BCL6 coding domain are resistant to CD40-mediated down-regulation (unpublished data). In these cases, constitutive BCL6 expression may contribute to lymphomagenesis by preventing CD80 expression and the functional B–T cell interaction which is necessary for post-GC differentiation of B cells into memory B cells or plasma cells. In addition, it is possible that lack of CD80 expression may prevent the recognition of lymphoma cells by tumor-specific T cells (32).

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