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Transcriptional Repression of Stat6-Dependent Interleukin-4-Induced Genes by BCL-6: Specific Regulation of Iε Transcription and Immunoglobulin E Switching

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The BCL-6 proto-oncogene encodes a POZ/zinc-finger transcription factor that is expressed in B cells and a subset of CD4$^+$ T cells within germinal centers. Recent evidence suggests that BCL-6 can act as a sequence-specific repressor of transcription, but the target genes for this activity have not yet been identified. The binding site for BCL-6 shares striking homology to the sites that are the target sequence for the interleukin-4 (IL-4)-induced Stat6 (signal transducers and activators of transcription) signaling molecule. Electrophoretic mobility shift assays demonstrate that BCL-6 can bind, with different affinities, to several DNA elements recognized by Stat6. Expression of BCL-6 can repress the IL-4-dependent induction of immunoglobulin (Ig) germ line ε transcripts, but does not repress the IL-4 induction of CD23 transcripts. Consistent with the role of BCL-6 in modulating transcription from the germ line ε promoter, BCL-6$^{-/-}$ mice display an increased ability to class switch to IgE in response to IL-4 in vitro. These animals also exhibit a multiorgan inflammatory disease characterized by the presence of a large number of IgE$^+$ B cells. The apparent dysregulation of IgE production is abolished in BCL-6$^{-/-}$ Stat6$^{-/-}$ mice, indicating that BCL-6 regulation of Ig class switching is dependent upon Stat6 signaling. Thus, BCL-6 can modulate the transcription of selective Stat6-dependent IL-4 responses, including IgE class switching in B cells.

Rearrangement of the BCL-6 proto-oncogene can be detected in 30 to 40% of diffuse large-cell lymphomas (DLCLs) and in 6 to 14% of follicular lymphomas (FLs) (5, 37, 42). In DLCLs and FLs, chromosomal rearrangements affecting the BCL-6 gene are located within a region spanning approximately 4 kb of the promoter and the first exon and result in the juxtaposition of the BCL-6 coding domains downstream of heterologous promoters derived from other chromosomes (53). These alterations lead to the production of chimeric transcripts which encode a wild-type BCL-6 protein, suggesting that the functional consequence of these translocations is the deregulation of BCL-6 expression by promoter substitution (53). The high frequency of dysregulated BCL-6 expression in these tumors suggests that this oncogene plays an important role in the transformation of human B cells.

The BCL-6 gene encodes a polypeptide containing six carboxy-terminal zinc-finger motifs homologous to members of the Krüppel subfamily of zinc-finger proteins (30, 38, 54). This domain of BCL-6 has been shown to recognize and bind to specific DNA sequences in vitro (4, 9, 48). The N-terminal portion of BCL-6 contains a ZiN (for zinc-finger N-terminal)/POZ (POX/zinc-finger) domain which is also present in other zinc-finger proteins, including the mammalian transcriptional regulators PLZF, ZF5, and ZID (3, 11, 12, 18, 40, 55). Transfection experiments have demonstrated that BCL-6 can act as a transcriptional repressor, and its ability to mediate repression requires the N-terminal POZ domain (9, 48). These results suggest that BCL-6 modulates transcription not simply through competitive binding, but through a mechanism of active repression. Indeed, the POZ domains of both BCL-6 and PLZF have recently been shown to associate with the SMRT corepressor, and, by extension, the histone deacetylase repression complex (17, 23, 25, 35).

BCL-6 is normally expressed in a tissue-specific and developmentally regulated manner. Although many tissues express low levels of BCL-6 mRNA, high levels of the BCL-6 protein have been found only in certain B cells and T cells (6). Within the B-cell lineage, BCL-6 is expressed at high levels in mature, germinal center B cells, but not in other B cells or plasma cells (6, 19, 41). BCL-6 expression in T cells is limited to cortical thymocytes and a population of CD4$^+$ cells within the germinal center and perifollicular zones of the lymph nodes (6). The importance of BCL-6 in normal lymphocyte function has recently been demonstrated in mice in which the gene for BCL-6 has been disrupted by homologous recombination (16, 20, 52). Although these mice contain normal numbers of B and T cells, they fail to form germinal centers or mount T-cell-dependent antibody responses. In addition, many of these mice develop a systemic inflammatory disease characterized by the infiltration of multiple organ systems by eosinophils and immunoglobulin E (IgE)-bearing B cells; these features are indicative of a Th2 polarized inflammatory response, which could potentially result from the inappropriate influence of the Th2 cytokines (interleukin-4 [IL-4], IL-5, and IL-13) on immune function. The striking phenotype of the knockout animal therefore

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implicates BCL-6 in the normal regulation of the immune system.

The evidence suggesting a disruption of cytokine regulation in the BCL-6$^{-/-}$ mice prompted the comparison between the in vitro defined binding site of BCL-6 (B6BS) and the binding sites of STAT proteins, molecules which are important mediators of cytokine signal transduction (reviewed in references 27, 34, and 46). In fact, B6BS shows a marked similarity to STAT recognition sequences, and one study has demonstrated the ability of BCL-6 to bind to the Stat6 site of the IL-4-inducible CD23b promoter (16). Furthermore, transient transfection studies have suggested that BCL-6 may regulate the Stat6-dependent transcription of the CD23b gene (16). However, the regulation of gene expression by BCL-6 under physiological conditions has not yet been tested, and other physiologic targets of BCL-6 repression are so far unknown.

In order to identify physiological targets for BCL-6, we have analyzed its ability to bind and regulate Stat6-dependent promoters in vitro and in vivo. Our results demonstrate that although BCL-6 can bind to the Stat6 sites present in several IL-4-responsive promoters in vitro, it can regulate only a subset of Stat6-dependent promoters in vivo; this subset includes the germ line ε promoter, but not the CD23b promoter. The germ line ε promoter regulates the production of the Ig sterile transcripts necessary for the Ig isotype class switch to IgE (reviewed in reference 13). Consistent with a role for BCL-6 in the regulation of class switching, IgE production is increased in B cells lacking BCL-6 in vitro and in vivo. This dysregulation of IgE production is not observed in B cells lacking Stat6 as well as BCL-6. These results provide evidence for the physiologic regulatory activity of BCL-6 on specific Stat6-dependent IL-4 signaling and identify the regulation of IgE class switching as a target of this activity in vivo.

MATERIALS AND METHODS

Mice. The BCL-6 knockout mice have been described previously (52). Stat6 knockout mice were obtained from Michael Grusby (29).

Plasmid construction. The eukaryotic expression vector pMT2T-BCL-6 and the reporter gene vector B6BS-LUC have been described previously (9). The reporter (luciferase) vector containing four copies of the Iε Stat6 binding site (14) was linked upstream to the minimal thymidine kinase (TK) promoter (Stat6-LUC). The reporter plasmid containing tandem repeats of human immunodeficiency virus (HIV)-κB binding motif linked upstream to the minimal murine c-*fos* promoter was obtained from D. Baltimore (10). The germ line ε driven reporter was constructed by insertion of Iε −162 to +57 into the *Mlu*I and *Bgl*II sites of the pGL2-basic vector (Promega). Germ line ε mutants were constructed by combining two PCR half-reactions with overlapping sequences, as described in (24). For the 5′ reaction, a common primer derived from −167 to −149 of the murine Iε promoter (mIε-5′, gggacgcgtCAGGTGTGTCTCCTAGAAA) was used with each of the following primers: S2m1, TCAACTCCTAGAAAGCAG AATCAAAAGGGAA; S2m4, TCAACTTCTAGAGAACAGAATCAAAAGG GAA; and S2m6, TCAACTTCCCGATCTCAGAATCAAAAGGGAA. For the 3′ reaction, a common primer derived from +42 to +55 of the murine Iε promoter (mIε-3′, tttagatctCCCCTGTGCAGGCT) was used with each of the following primers: S2m1, TTCTGCTTTCTAGGAGTTGACTAAGGCACAG; S2m4, TTCTGTTCTCTAGAAGTTGACTAAGGCACAG; and S2m6, TTCTG AGATCGGGAAGTTGACTAAGGCACAG. PCR half-reactions were then combined, and a second PCR was performed by using the common mIε-5′ and mIε-3′ primers. These primers were also used to generate a wild-type promoter construct (WT) (mIε −167 to +55). The products of these reactions were then cloned into the *Mlu*I and *Bgl*II sites of the pGL2-basic vector (Promega). All sequences were verified by automated sequencing at the DNA sequencing facility at Columbia University. The simian virus 40-chloramphenicol acetyltransferase (SV40-CAT) reporter plasmid driven by the CD23b promoter (−183 to −33) was obtained from D. Katz (43).

EMSA. Preparation of total cellular extracts from the Mutu I, Mutu III, and M12 lines was performed by an NP-40 lysis method as previously described (44). Electrophoretic mobility shift assays (EMSAs) and antibody-mediated supershift assays were performed as described previously (9), except that the EMSA reaction buffer was 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 10 mM $ZnCl_2$, 20 mM HEPES (pH 7.9), and 4% Ficoll. For competition assays, the indicated amount of cold competitor oligonucleotide was added 15 min prior to addition of labeled probe. The following probes were used in these experiments: Iε Stat6, 5′agctA ACTTCCCAAGAACA3′; CD23b Stat6, 5′GGTGAATTTCTAAGAAAGG3′; B6BS, 5′GAAAATTCCTAGAAAGCATA3′; S2m1, 5′gatcATTCTCTTTCTA GGAGTTGACTAAGGCACA3′; S2m4, 5′gatcATTCTGTTCTCTGGAAGTT GACTAAGGCACA3′; and S2m6, 5′gatcATTCTGAGATCGGGAAGTTGAC TAAGGCACA3′.

DNase I footprinting. Construction of the glutathione-*S*-transferase (GST)-BCL-6ZF expression plasmid has been previously described (39). For footprinting experiments, a germ line ε promoter fragment (−162 to +57) was end labeled on the noncoding strand and incubated (10,000 cpm) for 20 min at room temperature with the indicated amounts of purified recombinant proteins in 50 μl of binding buffer (40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 10 mM $ZnCl_2$, 20 mM HEPES [pH 7.9], 4% Ficoll). DNase footprinting was performed with the Pharmacia Biotech SureTrack Footprinting system, as per the manufacturer's protocol. The reaction products were ethanol precipitated, dried, and resuspended in 6 μl of loading dye. One-half of the sample (3 μl) was then loaded on a 6.3% wedge gel (0.4- to 1.2-mm gradient).

Cell lines, transient transfection, and reporter gene assays. The Mutu I and Mutu III cell lines were obtained from A. Rickinson (22) and maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 U of streptomycin per ml, 2 mM L-glutamine, and 10 mM HEPES. Mutu III cells (10^7) were transfected by electroporation with 4 μg of reporter constructs [B6BS-LUC, Stat6-LUC or κB(HIV)-LUC], 2 μg of β-galactosidase (β-gal) reporter plasmid, and the indicated amounts of the pMT2T-based expression constructs of either the full length of BCL-6 (BCL-6) or the zinc finger portion of BCL-6 (HAZF). The total DNA used in each transfection was adjusted to 30 μg by addition of control plasmids (pMT2T without cDNA insert). Cells were treated with 10 U of recombinant human IL-4 (Schering Plough) per ml or remained untreated immediately after transfection. After 24 h, the luciferase and β-galactosidase activities of the transfected cells were measured. Percent relative reporter gene activities were expressed as arbitrary units of luciferase activities normalized with β-galactosidase activities. M12.4.1 cells were maintained in RPMI supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 U of streptomycin per ml, 2 mM L-glutamine, and 1 mM β-mercaptoethanol. Cells (5×10^6) were transfected by electroporation as described previously (21). The transfection cocktail contained 5 μg of either germ line ε (−162 to +57) luciferase reporter, mutant germ line ε reporter luciferase reporter constructs (WT, S2m1, S2m4, or S2m6, as described above), or CD23b (−183 to −33) SV40-CAT reporter construct, 1 μg of β-gal reporter plasmid, and the indicated BCL-6 or HAZF expression constructs. Vector DNA (pMT2T) was added as necessary to achieve a constant amount of transfected DNA. Following transfection, cells were incubated in the presence or absence of 10 U of murine recombinant IL-4 per ml for 24 h. After 24 h, the luciferase activity of cells transfected with the germ line ε reporter was measured, while the CAT activity of those cells transfected with the CD23b reporter was measured at 36 to 48 h. Transfection efficiency was normalized relative to β-galactosidase activity.

LPS culture of IgM$^+$ B cells and Ig ELISA. Murine splenocytes were harvested as described previously (45) and enriched for IgM$^+$ cells by using anti-IgM magnetic beads (Miltenyi Biotec, Bergish-Gladback, Germany). After enrichment, the cells were found to be about 90% IgM$^+$ by fluorescence-activated cell sorter. The cells were cultured for 6 days with lipopolysaccharide (LPS) with or without cytokine (IL-4 or gamma interferon [IFN-γ]) as described previously (51). The concentration of secreted Ig of various isotypes was determined by enzyme-linked immunosorbent assay (ELISA). Anti-IgE antibodies were obtained from Pharmingen (San Diego, Calif.) and all of the other antibodies came from Southern Biotechnology Associates (Birmingham, Ala.). ELISA procedures were performed according to the manufacturer's recommendations.

Immunohistochemistry. A goat anti-mouse IgE polyclonal antibody (1:10,000; ICN Biomedicals, Aurora, Ohio) or a goat anti-mouse IgG1 polyclonal antibody (1:3,000; Southern Biotechnology Associates) was used to stain formalin-fixed, paraffin-embedded tissue sections after unmasking (7). Dewaxed sections were boiled in 10 mM EDTA (pH 7.5) for 15 min, cooled, blocked with 3% human AB serum (Sigma), and incubated overnight with the appropriate primary antibodies and control sera. The sections were then washed in washing buffer (Tris-buffered saline, 50 mM Tris [pH 7.5], 0.001% Tween 20) and counterstained with a 1:200 dilution of biotin-conjugated, mouse and human serum-adsorbed, rabbit anti-goat antibody (Southern Biotechnology Associates). Finally, horseradish peroxidase-conjugated avidin (Dako, Carpinteria, Calif.) was added and developed, after washing, with aminoethyl carbazole (Sigma). Slides were lightly counterstained with hematoxylin.

RESULTS

BCL-6 binds with high affinity to the Iε Stat6 site. The dysregulation of IgE production suggested by the increased number of IgE-bearing B cells present in the inflammatory infiltrate of BCL-6 null mice (52), compounded with the strong homology noted between the binding sites for STAT proteins and the in vitro defined binding site for BCL-6, led us to investigate the involvement of BCL-6 in the regulation of Stat

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signaling pathways. Binding sites for the Stat6 transcriptional activator are found in the regulatory regions of a number of IL-4-responsive genes. These include an element in the gene encoding IL-4 itself that has been reported to act as a silencer in Th1 cells (33), the germ line ε promoter (Iε) (14, 32, 47), and the promoter of the CD23b gene (31). One study has suggested that CD23b may be regulated by BCL-6 (16). In order to investigate the possibility of BCL-6 regulation of these genes, we first examined the ability of BCL-6 to bind Stat6 sites derived from these promoters in an EMSA. Extracts used in this assay were prepared from two Epstein-Barr virus-infected lymphoma lines that had been cultured either alone or in the presence of IL-4 for 1 h prior to harvest: Mutu I, which expresses BCL-6; and Mutu III, a BCL-6-negative line derived from the same patient (22). The extracts were incubated with probes generated from the Stat6 elements of either the Iε promoter, the CD23b promoter previously shown to bind BCL-6 (16), or the IL-4 silencer, and the products of the binding reaction were separated by polyacrylamide gel electrophoresis.

EMSA analysis of extracts prepared from the BCL-6-positive Mutu I line and incubated with probes derived from either the germ line ε or CD23b Stat6 sites reveals a single complex absent in EMSAs performed with extracts from the BCL-6-negative Mutu III cells (Fig. 1A and B). This constitutive complex is supershifted with an antiserum specific for BCL-6, but not with an antiserum which recognizes Stat6, confirming that this complex contains BCL-6. This band is also present in EMSAs performed with the IL-4 silencer Stat6 site as a probe (data not shown). Examination of extracts from IL-4-treated Mutu I cells by EMSA reveals an additional complex that is also present in EMSAs performed with extracts prepared from IL-4-treated Mutu III cells. Unlike the faster-migrating complex, which is supershifted specifically with the BCL-6 antiserum, this second complex is supershifted by the Stat6-specific antiserum and is present in EMSAs performed with all three Stat6 binding sites used as probes in these experiments. While the activated Stat6 complex is present at lower levels in EMSAs using extracts from Mutu III cells, it is unclear whether this phenomenon is due to the difference in BCL-6 expression, the stage of EBV infection, or another uncharacterized clonal variation between the two lines. These experiments indicate that BCL-6 can bind to the Stat6 sites present in the regulatory regions of many IL-4-responsive genes, including the Iε promoter, the CD23b promoter, and the putative silencer of the IL-4 gene.

In order to compare the relative affinity of BCL-6 for the Iε, B6BS (the in vitro defined binding site of BCL-6), CD23b, and IL-4 silencer binding sites, we analyzed the ability of unlabeled oligonucleotides derived from these various Stat6–BCL-6 sites to compete for BCL-6 binding with a labeled probe generated from the Stat6 site of the Iε promoter. Extracts prepared from cells of the murine B-lymphoma line M12.4.1 were incubated with labeled Iε probe and increasing concentrations of the appropriate cold competitor, and the reaction products were analyzed by EMSA (Fig. 1C and D). The results demonstrate that unlabeled Iε and B6BS are able to effectively compete with labeled probe for BCL-6 binding, even at the lowest concentrations used in this assay (Fig. 1C, lanes 2 to 5 and 7 to 10). The Stat6 binding sites from the CD23b promoter and IL-4 silencer, however, prove considerably less effective competitors of BCL-6 binding. While they compete for BCL-6 binding at the highest concentrations used in this assay (Fig. 1D), the affinity of these sites for BCL-6 seems weak relative to that of Iε or B6BS. These data indicate that BCL-6 can bind to some sites also recognized by Stat6, but that its affinity for these sites is not equivalent. Furthermore, the previously defined Stat6 element of the germ line ε promoter appears to be a high-affinity binding site for BCL-6.

In order to further characterize the relationship between the Stat6 and BCL-6 recognition sites of the germ line ε promoter, we examined the regions of Iε these proteins protect from DNase I cleavage in footprinting studies. A purified GST fusion with the zinc finger DNA binding domain of BCL-6 and purified recombinant Stat6 protect overlapping regions of the germ line ε promoter (Fig. 2). The region protected by BCL-6 is completely contained within that protected by Stat6 and includes the previously described Stat6 site at −111 to −102 relative to the transcriptional start site (14). As these experiments were performed with only the DNA binding domain of BCL-6, it is possible that the full-length protein may protect a larger region than is indicated here. Nevertheless, it is clear from these studies that BCL-6 and Stat6 share a common binding site at the germ line ε promoter.

BCL-6 represses transcription from an Iε-driven promoter. Previous studies have demonstrated the ability of BCL-6 to act as a site-specific transcriptional repressor in transient transfection assays (9, 48). In order to assess the functional significance of BCL-6 binding to the germ line ε promoter, we first sought to determine the effect of BCL-6 expression on the IL-4-induced transcription of a reporter driven by the upstream Stat6 element of the Iε promoter (−115 to −99). A tetramer of this site was placed upstream of the minimal TK promoter and a luciferase reporter; this construct was cotransfected with increasing amounts of a BCL-6 expression vector into the BCL-6-negative Mutu III human B-cell line. Transfected cells were cultured either alone or in the presence of IL-4 for 24 h, at which time they were harvested and assayed for luciferase activity. As shown in Fig. 3A, IL-4 treatment results in an approximately 20-fold increase in luciferase activity from the Iε-Stat6-LUC reporter. This cytokine-induced activation of the luciferase reporter is blocked by BCL-6 in a dose-dependent fashion, as increasing concentrations of BCL-6 lead to a 75% decrease in the maximal IL-4-induced luciferase activity. BCL-6 similarly represses transcription from a luciferase reporter driven by the cytokine-independent in vitro defined binding site of BCL-6 (B6BS-LUC) (Fig. 3B). Cotransfection of BCL-6 with an irrelevant reporter, such as the HIV-κB-driven luciferase construct shown in Fig. 3C, does not result in the reduction of reporter signal, demonstrating the specificity of BCL-6 repression.

The BCL-6 POZ domain has been shown to interact with the

FIG. 1. The germ line ε promoter is a high-affinity binding site of BCL-6. Two EBV-transformed human B-cell lines which differentially express BCL-6, Mutu I (BCL-6 positive) and Mutu III (BCL-6 negative), were cultured in the presence or absence of human recombinant IL-4 (10 U/ml) for 1 h prior to harvest. EMSAs were performed with 5 μg of whole-cell extract and an oligonucleotide probe corresponding to the germ line ε promoter Stat6 site (A) or the CD23b Stat6 site (B). The DNA binding complexes were identified upon supershift with antisera (Ab [antibody]) to the Stat6 (αStat6) or BCL-6 (αBCL-6) proteins. In panels C and D, the affinity of BCL-6 for the Stat6 site at −115 to −99 of the germ line ε promoter relative to other known binding sites of BCL-6 was assessed in cold competition assays. Five micrograms of whole-cell extract prepared from the BCL-6-positive M12.4.1 murine B-cell lymphoma line was incubated with a labeled probe generated from the Iε Stat6 site (−115 to −99) and increasing concentrations (10, 30, 60, or 100 ng) of cold competitor oligonucleotides. (C) The Iε Stat6 site (lanes 2 to 5) and B6BS, the in vitro defined binding site for BCL-6 (lanes 7 to 10). (D) The Iε Stat6 site (lanes 2 to 5), the Stat6 site of the CD23b promoter (lanes 7 to 10), and the Stat6 site of the putative IL-4 silencer (lanes 12 to 15).

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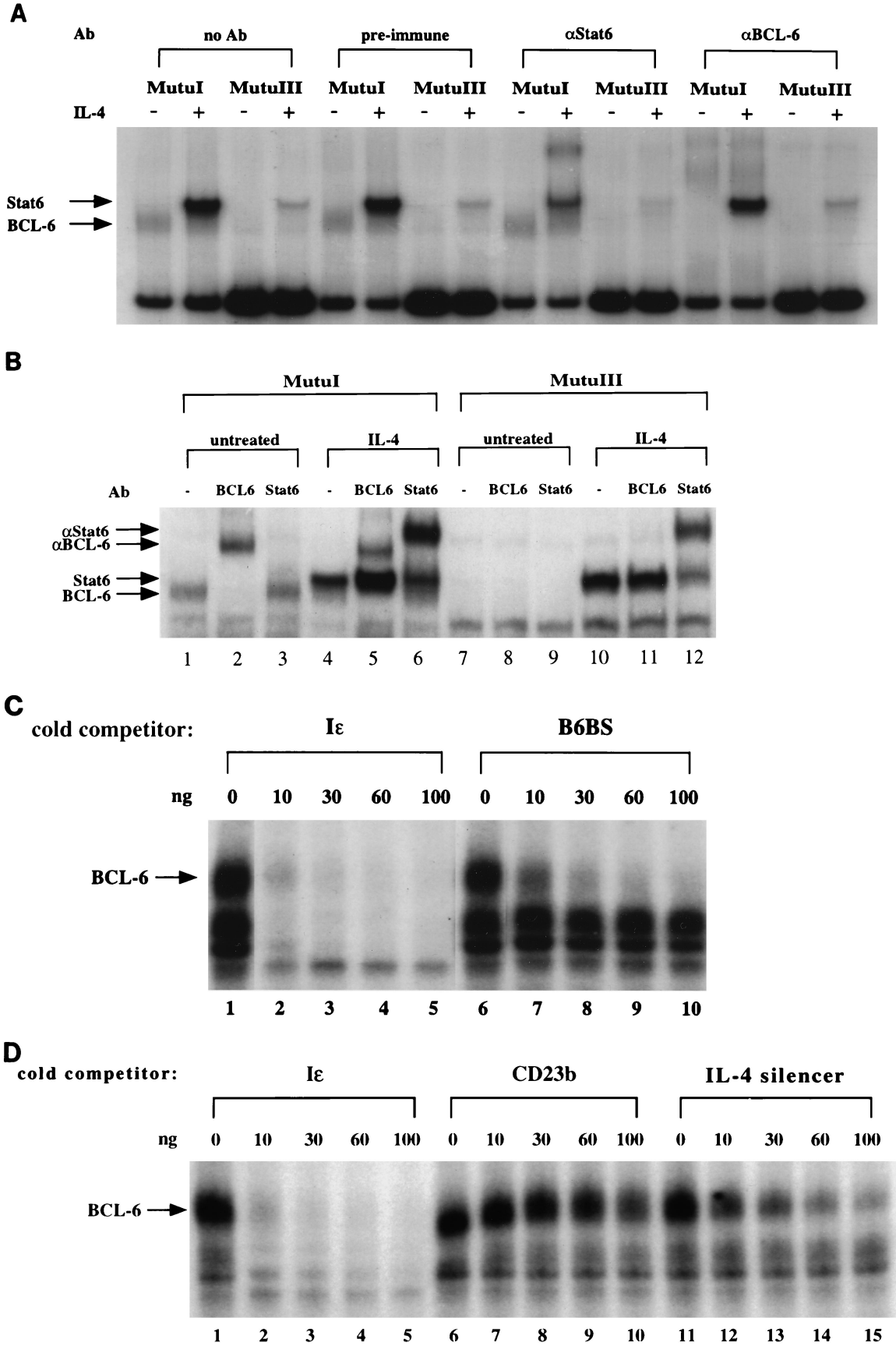

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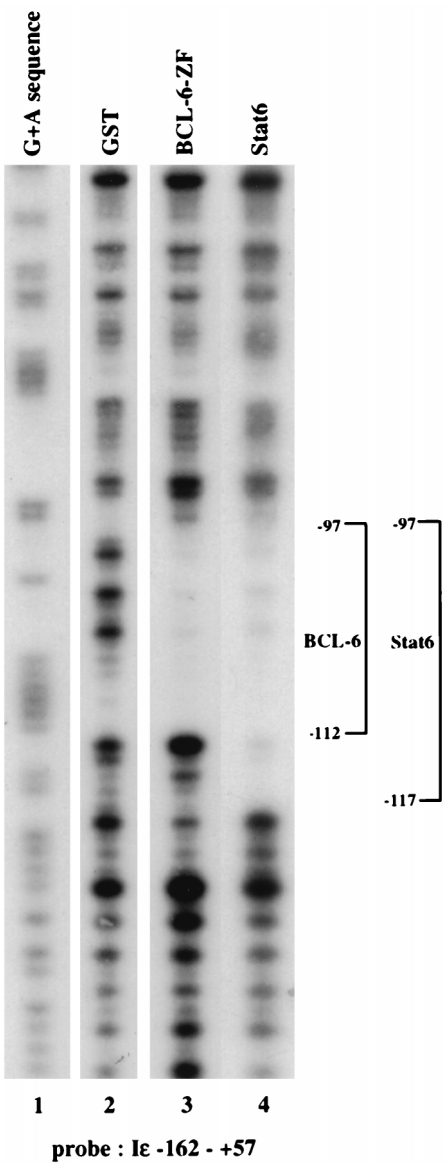

FIG. 2. BCL-6 and Stat6 protect overlapping regions of the germ line ε promoter. Binding of purified GST (400 ng) (lane 2), recombinant Stat6 (100 ng) (lane 3), or a fusion protein of GST with the DNA binding domain of BCL-6 (400 ng) (lane 4) to the murine germ line ε promoter was compared by DNase I footprinting analysis. A G+A sequencing reaction is shown in lane 1. The positions of the protected regions relative to the transcriptional start site (13) are indicated.

SMRT corepressor and is required for BCL-6 to effectively mediate repression (9, 17, 48). Nevertheless, it remains possible that competition for any of the four Iε-Stat6 sites of the Iε-Stat6-LUC reporter contributes to the ability of BCL-6 to repress Stat6-mediated transcription in these transfection assays (although the affinity of BCL-6 for these sites is less than that of Stat6 [unpublished observations]). This model would predict that a mutant BCL-6 protein lacking the POZ repressor domain might retain some ability to repress IL-4-induced transcription—through direct competition with Stat6 for a common binding site—so long as its DNA binding domain remains intact. This truncated form of BCL-6 (HAZF) binds B6BS and the Iε Stat6 site with the same affinity as the wild-type molecule (reference 48 and data not shown). However, HAZF is unable to repress the IL-4 induction of the Iε-Stat6 reporter construct in cotransfection experiments (Fig. 3D). These results imply that the binding of BCL-6 to Stat6 sites is not sufficient to repress Stat6 activity and indicate that the BCL-6-mediated repression of Stat6 function is active, requiring the POZ repressor domain of BCL-6.

Although we had demonstrated the ability of BCL-6 to repress the activity of a reporter driven by a multimerized Iε-Stat6 site, we wanted to determine if BCL-6 was effective in repressing IL-4-induced transcription in the context of a larger segment of the germ line ε promoter. The region of murine Iε spanning from −162 to +57, which includes the binding sites for many of the factors known to regulate the activity of this promoter, was used to drive the transcription of a luciferase reporter. This construct was cotransfected with increasing amounts of a BCL-6 expression vector into cells of the M12.4.1 murine B-lymphoma line. Transfected cells were cultured either alone or in the presence of IL-4 for 24 h and then harvested and assayed for luciferase activity. As shown in Fig. 4A, the IL-4-induced activity of this reporter construct is repressed by BCL-6 in a dose-dependent manner. The truncated HAZF form of BCL-6, however, is unable to repress transcription in these assays; in fact, cotransfection of HAZF appears to result in an increased basal level of transcription of this reporter, perhaps due to binding competition with the endogenous BCL-6. Interestingly, in similar experiments performed with a CAT reporter driven by the IL-4-responsive region of the murine CD23b promoter (−183 to −33) (43), BCL-6 failed to mediate the repression of IL-4-induced transcription (Fig. 4B). These experiments demonstrate the ability of BCL-6 to modulate the Iε transcriptional response to IL-4, not only when expression is driven by a multimerized Stat6 element derived this site, but also when driven by a large segment of the germ line ε promoter itself. In addition, the regulation of transcription by BCL-6 appears to be limited to a subset of IL-4-inducible promoter regions.

Repression of germ line ε transcription is dependent upon BCL-6 binding at Iε −111 to −102. The experiments described above have demonstrated the ability of BCL-6 to bind to the Stat6 site at −111 to −102 of the murine Iε promoter and the ability of BCL-6 to repress transcription from this promoter in transient transfection assays. However, it remained possible that the repression mediated by BCL-6 occurs through a mechanism other than the direct binding of BCL-6 to this shared site. In order to formally discount this possibility, we created a series of mutants in which the binding of either BCL-6, Stat6, or both proteins to the BCL-6–Stat6 site is disrupted (Fig. 5A). These mutations were generated within the context of the IL-4-responsive region of the murine germ line ε promoter (−167 to +55) and specifically ablate the association of the targeted protein with its binding site.

EMSA analysis using oligonucleotides derived from the various binding site mutants as probes exhibited the predicted patterns of BCL-6 and Stat6 protein binding (Fig. 5B). S2mut1 fails to bind activated Stat6 present in extracts from IL-4-stimulated M12 cells, but retains the ability to bind BCL-6 present in these extracts. Conversely, S2mut4 cannot bind BCL-6 present in M12 cell extracts, yet retains its ability to bind Stat6 present in extracts from IL-4-stimulated M12 cells. S2mut6 is unable to bind either protein (Fig. 5B). We next analyzed the ability of unlabeled mutant Stat6–BCL-6 promoters to compete for factor binding with the wild-type Iε promoter (Fig. 5C). These results demonstrate that the affinities of proteins not targeted by the specific mutations are little changed. In addition, the binding of the transcription factor C/EBPβ to its adjacent site at −120 to −113 is undisturbed (data not shown).

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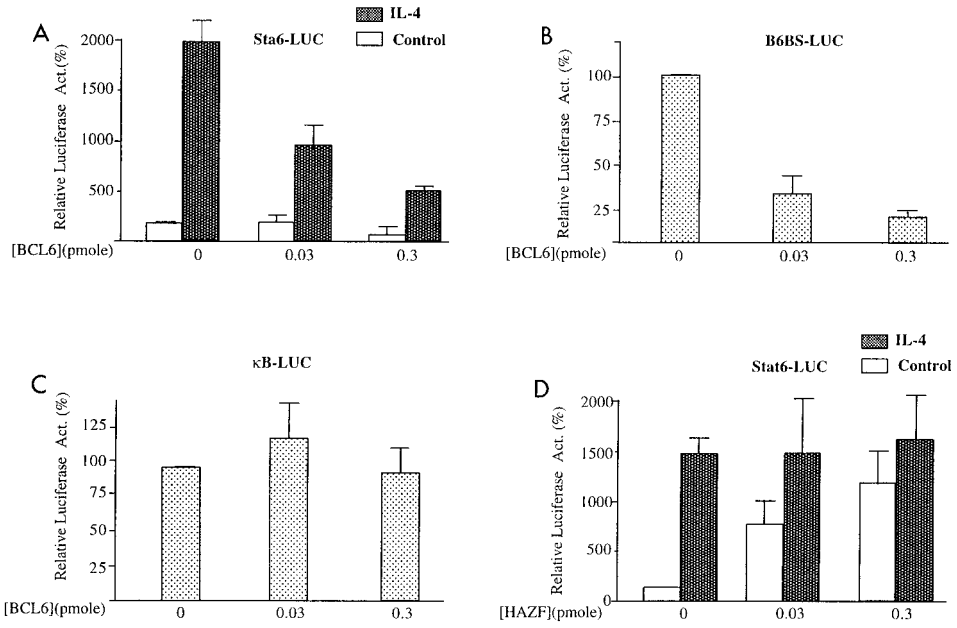

FIG. 3. BCL-6 represses IL-4-inducible transcriptional activity. Mutu III cells (10^7) were transfected with TK-luciferase reporter constructs driven by multimerized binding sites corresponding to either the Iε Stat6 site (Stat6-LUC) (A and D), the in vitro defined binding site of BCL-6 (B6BS-LUC) (B), or an HIV-κB binding site linked upstream of the minimal c-Fos promoter (κB-LUC) (C). These cells were cotransfected with increasing amounts of plasmid expressing either the full length BCL-6 (A to C) or the zinc finger DNA binding domain of BCL-6, HAZF (D). Following electroporation, the cells were either left untreated (B and C) or were divided and cultured either alone or in the presence of IL-4 (10 U/ml) for 24 h (A and D). Percent relative reporter gene activities are expressed as arbitrary units of luciferase activities normalized with β-galactosidase activities. The results are given as means ± standard deviations from three separate experiments.

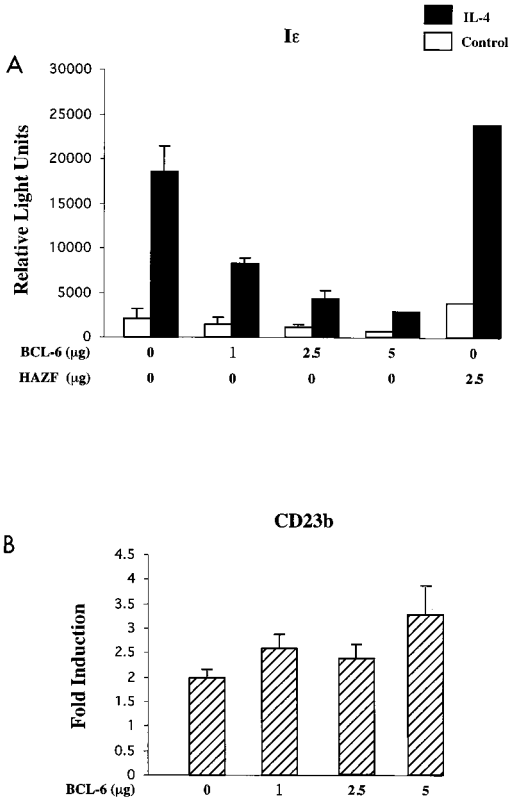

FIG. 4. Differential regulation of Iε and CD23b by BCL-6. M12.4.1 cells (5×10^6) were cotransfected with a luciferase reporter driven by either −162 to +57 of the murine germ line ε promoter (A) or −183 to −33 of the murine CD23b promoter (B) and increasing concentrations of the indicated expression construct. Following electroporation, the cells were divided and cultured either alone or in the presence of IL-4 (10 U/ml) for 24 to 48 h. The results are given as means ± standard deviations from two (Iε) or three (CD23b) separate experiments and are normalized with β-galactosidase activities. Fold induction is given relative to unstimulated cells.

In order to assess the dependence of BCL-6-mediated repression on the presence of an intact BCL-6 binding site at Iε −111 to −102, the promoter mutants described above were used to drive a luciferase reporter in transient transfection assays. These constructs were cotransfected with either a control plasmid or with a BCL-6 expression vector into cells of the M12.4.1 murine B-lymphoma line. Transfected cells were cultured either alone or in the presence of IL-4 for 24 h and then harvested and assayed for luciferase activity. As expected, germ line ε promoter constructs with mutations in their Stat6 binding sites (S2mut1 and S2mut6) are unresponsive to stimulation by IL-4 (Fig. 5D). These promoters are little affected by cotransfection with BCL-6. On the other hand, promoter constructs with intact Stat6 binding sites demonstrate equivalent induction of transcription in response to IL-4, regardless of BCL-6 binding activity (WT and S2mut4). Significantly, while IL-4 induction of wild-type promoter activity is repressed by cotransfection of BCL-6 in these experiments, BCL-6 is unable to mediate the repression of IL-4-induced transcription from the promoter carrying the mutant BCL-6 binding site (S2mut4). These results demonstrate the requirement of an intact BCL-6 binding site at Iε −111 to −102 for the proper function of the repressor.

Class switching to IgE is increased in B cells from BCL-6$^{-/-}$ mice. Examination of mice with a targeted disruption of BCL-6 has revealed several interesting phenotypes, including a lack of germinal centers and a novel Th2-type inflammatory disease

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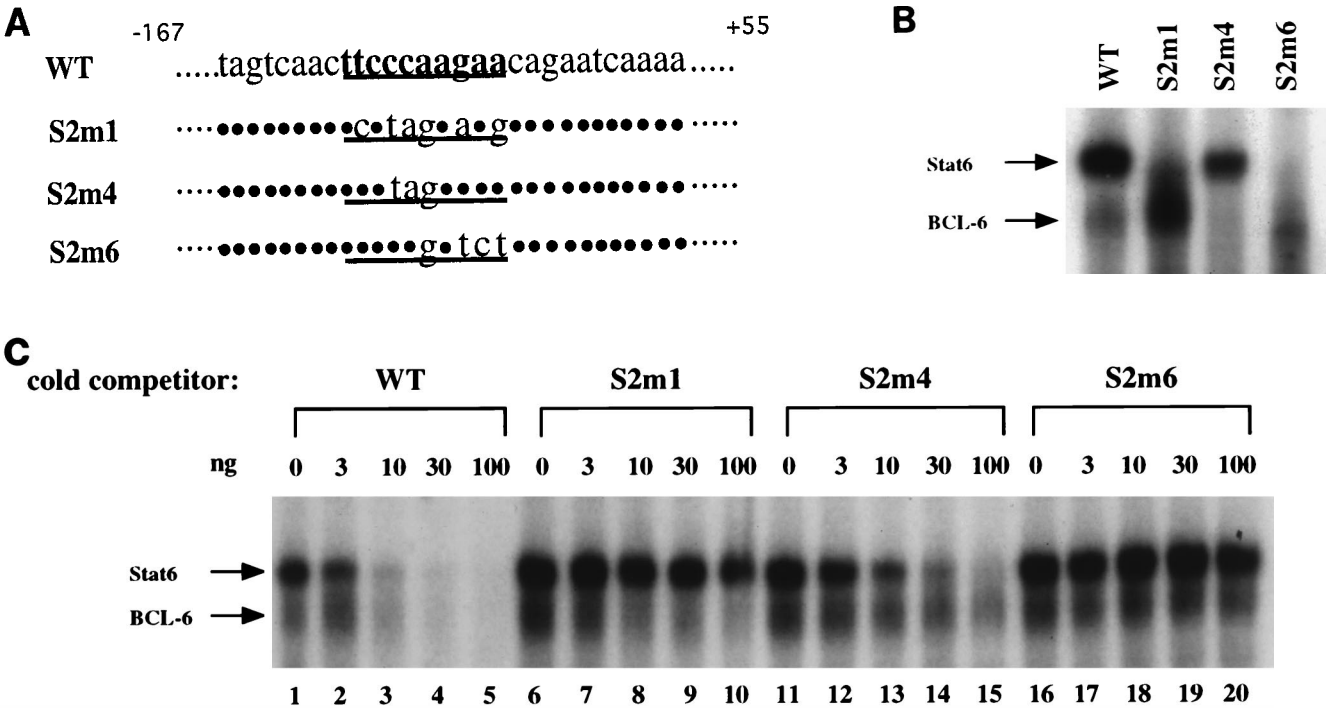

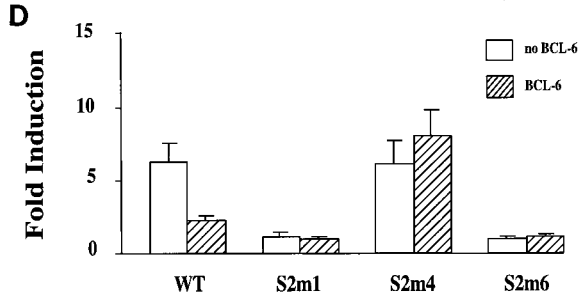

FIG. 5. Repression of germ line ε transcription is dependent on an intact BCL-6 binding site. (A) Schematic diagram of mutations used in this study. These mutations were generated within the context of the germ line ε promoter (−167 to +55). (B) M12.4.1 cells were cultured in the presence of recombinant murine IL-4 for 1 h prior to harvest. EMSAs were performed with 5 μg of whole-cell extract and oligonucleotide probes corresponding to the germ line ε promoter BCL-6–Stat6 site mutants described in panel A: WT (lane 1), S2m1 (lane 2), S2m4 (lane 3), and S2m6 (lane 4). In panel C, the affinity of BCL-6 for the wild-type BCL-6–Stat6 site relative to the mutant BCL-6–Stat6 binding sites was assessed in cold competition assays. Five micrograms of whole-cell extract prepared in panel B was incubated with a labeled probe generated from the wild-type BCL-6–Stat6 site and increasing concentrations (3, 10, 30, or 100 ng) of cold competitor oligonucleotides: the wild-type BCL-6–Stat6 site (lanes 2 to 5), S2m1 (lanes 7 to 10), S2m4 (lanes 12 to 15), and S2m6 (lanes 17 to 20). In panel D, 5×10^6 M12.4.1 cells were cotransfected with a luciferase reporter driven by the indicated variations on the germ line ε promoter (WT, S2m1, S2m4 or S2m6) and either control plasmid or 2.5 μg of BCL-6 expression vector. Following electroporation, the cells were divided and cultured either alone or in the presence of IL-4 (10 U/ml) for 24 to 48 h. The results are given as means ± standard deviations from three separate experiments and are normalized with β-galactosidase activities. Fold induction is given relative to unstimulated cells.

characterized by the infiltration of multiple organ systems by eosinophils and IgE^+ B cells (16, 52). The presence of these IgE^+ B cells within the inflammatory infiltrate observed in BCL-6-deficient mice suggests that there is some dysregulation of IgE production in mice that lack BCL-6. The data presented above demonstrate that the expression of BCL-6 in B lymphocytes can inhibit the induction of germ line ε transcription in response to IL-4. Because the IL-4-dependent production of germ line ε transcripts is a requisite step in the process of Ig class switching to IgE, it is possible that BCL-6 normally performs a role in regulating this process by inhibiting the induction of germ line ε transcription by IL-4.

In order to determine whether the effect of BCL-6 on germ line ε transcription has physiologic significance in vivo, we examined Ig class switching in B cells that lack expression of BCL-6. IgM^+ B cells were isolated from the spleens of BCL-$6^{-/-}$ mice and wild-type littermate controls and then cultured with the mitogen LPS for 6 days, either alone or in the presence of IL-4 or IFN-γ. The ability of these cells to class switch to different Ig isotypes was measured by ELISA analysis of the supernatants of these cultures. As shown in Fig. 6, the production of IgM and IgG2a by B cells isolated from wild-type or BCL-6-deficient mice is not significantly different in this in vitro culture system. In contrast, the ability of cells derived from the BCL-$6^{-/-}$ mice to produce IgE and IgG1 (another IL-4-dependent Ig isotype) in response to IL-4 is markedly enhanced when compared with that of cells isolated from wild-type controls, supporting an in vivo role for BCL-6 in the regulation of class switching.

BCL-$6^{-/-}$ Stat$6^{-/-}$ mice do not show increased class switching to IgE. Although we had shown that B cells derived from BCL-$6^{-/-}$ mice demonstrate a marked enhancement in IgE production, it remained conceivable that the dysregulation of IgE responses observed in these animals was due to a disruption in the regulation of pathways other than those activated through Stat6 signaling. In order to formally prove that BCL-6 deregulation of Iε transcription acts on a Stat6-dependent pathway, we generated mice doubly deficient in BCL-6 and Stat6. Like the BCL-$6^{-/-}$ animals, the BCL-$6^{-/-}$ Stat$6^{-/-}$

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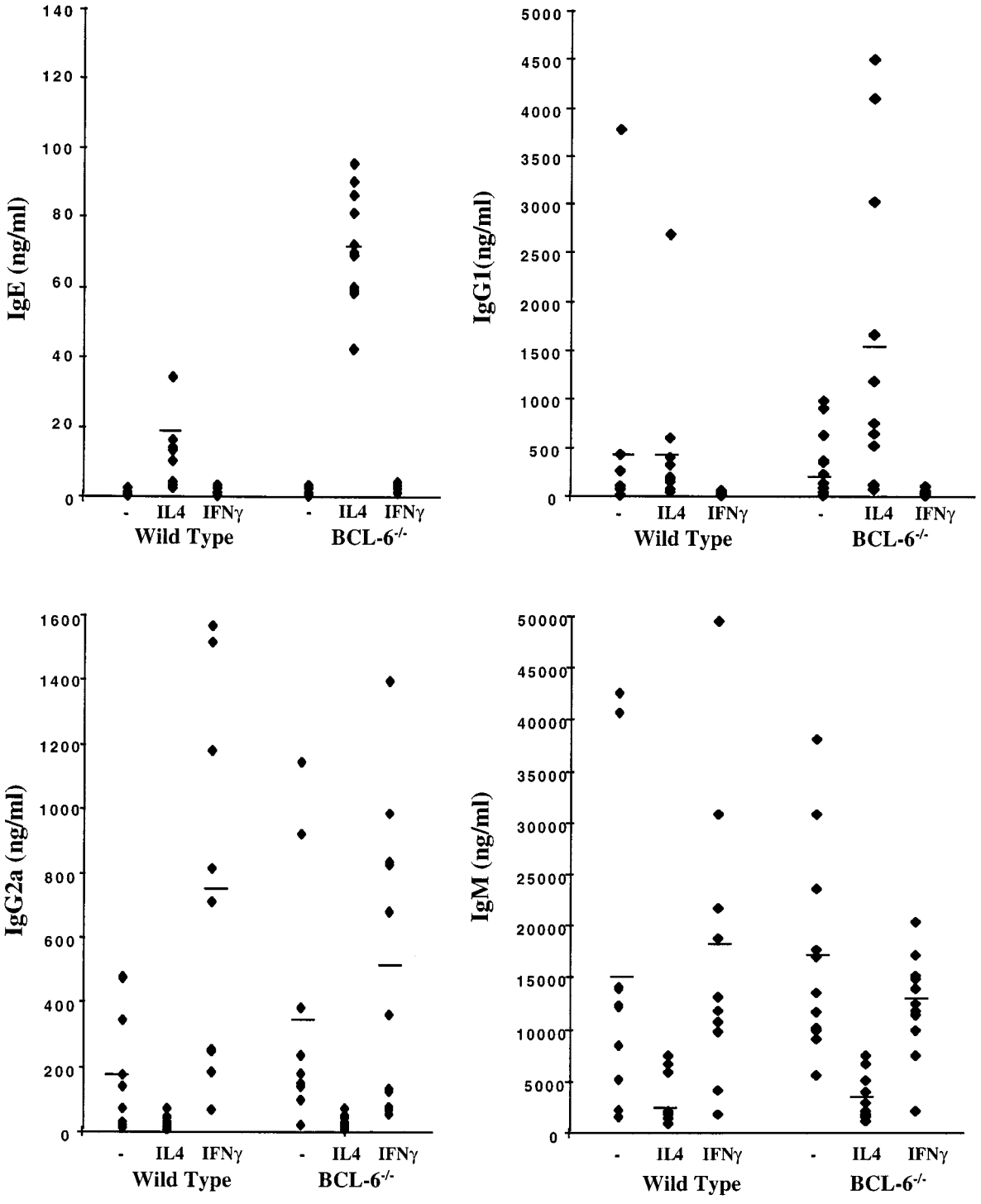

FIG. 6. B cells from BCL-6$^{-/-}$ mice produce higher levels of IgE in response to IL-4. IgM^{+} B cells from the spleens of 11 BCL-6$^{-/-}$ mice or 10 wild-type littermates were cultured in the presence of LPS and cytokine (either 5,000 U of IL-4 per ml or 60 U of IFN-γ per ml) for 6 days. Supernatants from these cultures were assayed for secreted Ig isotypes by ELISA. Each diamond represents the levels from 1 mouse. The mean concentrations are noted by lines.

mice, although born according to Mendelian frequencies, are runted, lack germinal centers, and exhibit a multiorgan inflammatory disease (8a). However, the inflammatory infiltrate present in the BCL-6$^{-/-}$ Stat6$^{-/-}$ mice does not contain the IgE^{+} B cells which characterize the disease of the BCL-6$^{-/-}$ parental strain (Fig. 7A). Analysis of these animals confirms that the increased level of IgE and IgG1 production observed in mice deficient in BCL-6 is dependent on Stat6 signaling pathways, as LPS–IL-4 cultures of IgM^{+} B cells isolated from BCL-6$^{-/-}$ Stat6$^{-/-}$ mice are greatly deficient in class switching to either IgE or IgG1 (Fig. 7B). These data provide a genetic demonstration of the modulation of Stat6 function by BCL-6

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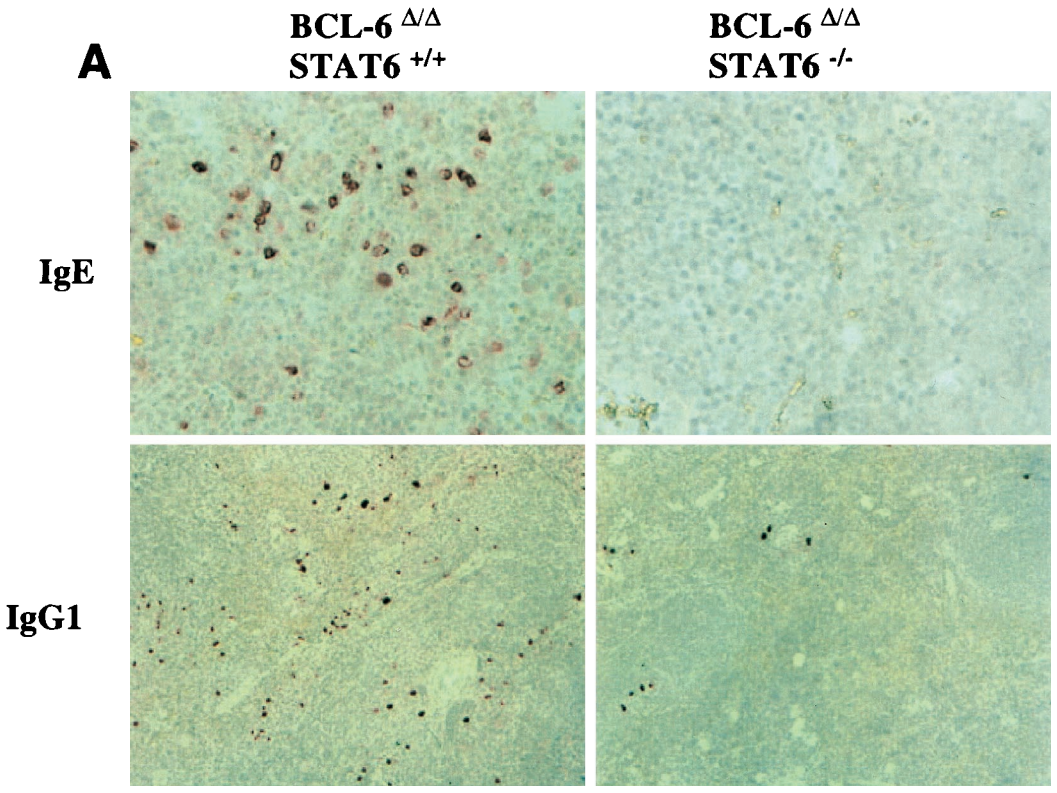

FIG. 7. BCL-6$^{-/-}$ Stat6$^{-/-}$ mice do not produce increased levels of IgE. (A) Submandibular lymph node (top) and spleen (bottom) sections from BCL-6$^{\Delta/\Delta}$ Stat6$^{+/+}$ (left panels) or BCL-6$^{\Delta/\Delta}$ Stat6$^{-/-}$ mice (right panels) were stained with anti-IgE or anti-IgG1 mouse antibodies, as indicated. The original magnifications were ×125 (top) and ×80 (bottom). (B) IgM^{+} B cells were isolated from the spleens of BCL-6$^{-/-}$ mice, Stat6$^{-/-}$ mice, BCL-6$^{-/-}$ Stat6$^{-/-}$ mice, or wild-type littermate controls. LPS cultures and ELISAs were performed as in Fig. 6. Each diamond represents the levels from one mouse. The mean concentrations are noted by lines.

and therefore further support an in vivo role for BCL-6 in the regulation of the IgE immune response.

DISCUSSION

This study was aimed at identifying the physiologically relevant transcriptional events regulated by the BCL-6 proto-oncogene. The main findings of the study are that BCL-6 is likely to modulate the transcription of some, but not all, Stat6-dependent genes. In particular, our results identify the Iε promoter as a physiologic target of BCL-6 transcriptional regulation. These results have implications for the function of BCL-6, the mechanism regulating IgE switching, and the role of BCL-6 in lymphomagenesis.

Selective modulation of Stat6-dependent transcription by BCL-6. Previous studies have demonstrated the recognition of Stat6 binding elements by BCL-6 and suggested that BCL-6 may broadly modulate Stat6-mediated IL-4 signaling (16). However, we show here that despite the fact that BCL-6 can bind various Stat6 binding sites in vitro and regulate the corresponding promoters under highly experimental conditions, its physiologic role in regulating Stat6-mediated transcription may be more specific. This conclusion is supported by the following observations: (i) BCL-6 can bind various Stat6 sites with different affinity in vitro (Iε > IL-4 > CD23b), (ii) BCL-6 can regulate the transcription of only a subset of IL-4-inducible genes in transiently transfected cells (Iε, but not CD23b), and (iii) the lack of BCL-6 deregulates IgE class switching in vivo. Thus, our results do not support a broad role for BCL-6 in the regulation of Stat6-dependent IL-4 signaling and, in particular, indicate that CD23b may not be a target of BCL-6 in vivo. The discrepancy between our data and results previously obtained with the CD23b promoter may be explained by the observation that the high levels of BCL-6 typically introduced through transient transfection were in this instance used to examine the effect of BCL-6 on a similarly overexpressed reporter; in previous studies, however, transient transfection assays were used to examine the effect of BCL-6 on the activation of the endogenous CD23b gene (16). However, we cannot entirely discount the possibility that CD23b transcription is regulated, at some level, by BCL-6, for although increasing concentrations of BCL-6 had no effect on the levels of IL-4-induced transcription in our system, a slight repression of basal transcription of the CD23b reporter was observed at the highest dosage of BCL-6 (5 μg) used in our cotransfection assays. This is reflected in the modest increase in fold induction seen when higher levels of BCL-6 are cotransfected with the CD23b reporter (Fig. 4B). It should be noted, however, that in similar studies using the germ line ε reporter, cotransfection of even low levels of BCL-6 (1 μg) resulted in a significant repression of basal, as well as IL-4-inducible, transcription.

The mechanism for the selective activity of BCL-6 is not known. It is possible that BCL-6 plays a role in determining the activation thresholds of various IL-4-responsive genes, repressing basal transcription from promoters which bind BCL-6 over a wide range of affinities, yet repressing activated transcription only from sites to which it binds avidly. Alternatively, the ability of BCL-6 to repress transcription may be more dependent on promoter context or topology. For instance, the presence of certain promoter-specific transcription factors or

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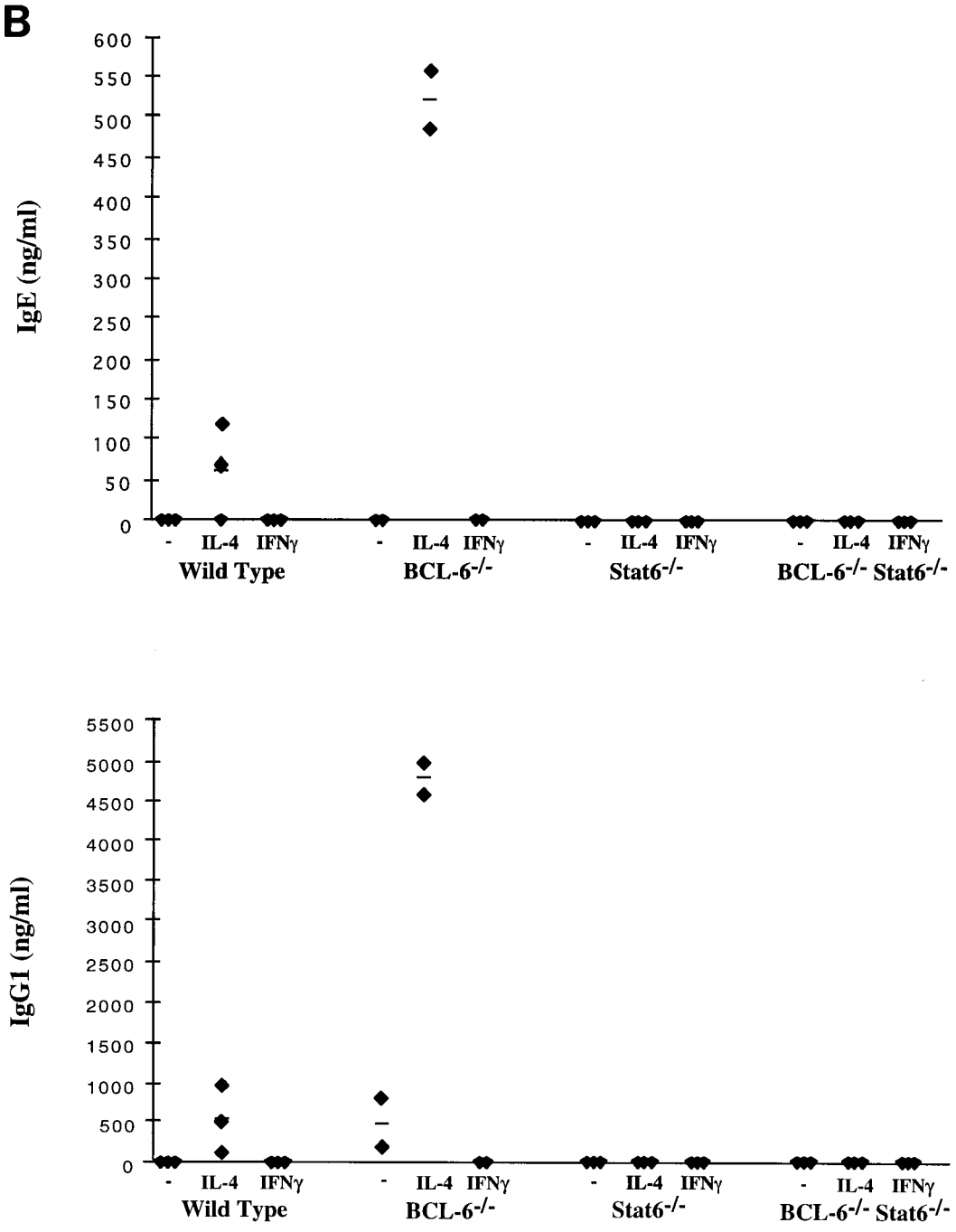

FIG. 7—*Continued.*

additional Stat6 binding sites may allow BCL-6-mediated repression of germ line ε transcription to the exclusion of CD23b. In this regard, recent studies involving the exchange of high- and low-affinity binding sites of the B-cell-specific activator protein (BSAP) have demonstrated that the context of a particular binding site can be more important than its relative affinity for BSAP in determining the activity of promoters regulated by this transcription factor (49).

Modulation of Iε transcription and IgE switching by BCL-6. Our results clearly identify Iε transcription as a physiologic target of BCL-6. The induction of germ line ε transcription in germinal center B lymphocytes is one important mechanism by which IL-4 regulates the production of IgE (13). Mice which are deficient in Stat6 do not secrete IgE in response to IL-4 stimulation, presumably due to their inability to produce these transcripts (36). By extension, the enhanced production of IgE by B cells which lack BCL-6 may be attributed to the absence of BCL-6 repressive activity at the Iε promoter. In support of this argument, we have demonstrated the high-affinity binding of BCL-6 to the Iε Stat6 site, the ability of BCL-6 to mediate the repression of germ line ε transcription in transient transfection assays, and the dependence of repression on an intact BCL-6 site within the germ line ε promoter. We have further shown that the hyper-IgE response observed in the BCL-6

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knockout mice is absent in animals doubly deficient in BCL-6 and Stat6, illustrating the dependence of the BCL-6$^{-/-}$ phenotype on Stat6 with respect to IgE production. These experiments represent the first evidence that BCL-6 can in fact regulate Stat6-inducible gene expression in vivo. Previous studies have failed to demonstrate any reciprocal regulation of gene activity by BCL-6 and Stat6, as illustrated by the perseverance of BCL-6$^{-/-}$ phenotypes, such as hyperinflammation and Th2 shift, in mice deficient in Stat6 as well as BCL-6 (15). However, despite convincing evidence implicating BCL-6 in the regulation of germ line ε transcription, significant differences in the levels of the IL-4-induced Iε transcript were not detected between the B cells of BCL-6$^{-/-}$ mice and wild-type controls (data not shown). This result may be explained by our finding that only about 5% of splenic B cells, i.e., germinal center B cells, express detectable levels of the BCL-6 protein (6); it is unlikely that changes in the production of germ line ε transcript would be detected in this small fraction of the B-cell population. In addition, it is possible that BCL-6 has a direct effect on the ability of these cells to secrete IgE. Recent data suggest that induction of IgE by IL-4 occurs both through increased class switching to IgE and through the increased secretion of IgE by cells which have switched to production of this isotype (48a).

Two Stat6 binding elements have been identified within the IL-4-responsive region of the germ line ε promoter; mutations made at either site can result in an increase in Iε basal transcription, suggesting a disruption in the recruitment of a repressor to these sites (1, 14, 26, 50). The data presented in this study demonstrate that BCL-6 can, in fact, bind to the Stat6 element at −111 to −102 of the germ line ε promoter and repress transcription from this promoter. There are several cases in which transcriptional activators and repressors have been described to recognize common DNA elements; in many of these instances, the mechanism of repression is passive, involving simple competition for the shared binding motif (reviewed in reference 28). However, results from our transfection studies using a truncated form of BCL-6 suggest that the repression of Stat6 activity mediated by BCL-6 requires the intact BCL-6 POZ repression domain. Because the truncated form of BCL-6 binds DNA avidly (48), these data imply that the repression of Stat6 function occurs through active repression and not only competitive binding. These results correlate with data which have demonstrated the interaction of the SMRT corepressor complex with the POZ domain of BCL-6 (17). Given these data, it is likely that the regulation of germ line ε transcription by BCL-6 is directed through its site-specific binding to either of the two Stat6 elements of the Iε promoter and is mediated through the recruitment of corepressors to the promoter.

The role of BCL-6 in the regulation of germinal center events, such as Ig class switching, is indicated by its restricted expression within the lymphocyte population to germinal center B cells and T cells. In fact, BCL-6 is required for the formation of germinal centers (16, 20, 52), suggesting a model of BCL-6 function in which the repressor complexes containing BCL-6 are already in place when the B cell enters the cytokine-rich culture of the germinal center. In this environment, the protection provided by BCL-6 against differentiative signals is alleviated only with the convergence of multiple stimuli which cooperate to relieve the BCL-6-mediated repression. Such stimuli may include signaling through the antigen receptor and CD40, which have been shown to regulate levels of BCL-6 protein and mRNA, respectively (2, 8, 39), and the IL-4-induced activation of Stat6, which can compete directly for BCL-6 binding and may efficiently displace it from the promoter. In this manner, BCL-6 can modulate transcription from its targets, repressing the activity of promoters such as Iε until an activation threshold is achieved and the balance of regulatory forces shifts in favor of transcriptional activation.

Implications for the role of BCL-6 in lymphomagenesis. Deregulated BCL-6 expression caused by chromosomal translocation is observed in 30 to 40% of DLCLs and in 5 to 10% of FLs. Both DLCLs and FLs derive from GC B cells and can be considered aberrations of GC development. Since cytokines play a key role in the proliferation and differentiation of B cells within the germinal center, the effect of BCL-6 in modulating specific cytokine signaling suggests that the inappropriate expression of BCL-6 might prevent certain cytokine-induced events required for the normal differentiation of germinal center cells and thereby contribute to the transformation process.

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