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BCL-6 Represses Genes that Function in Lymphocyte Differentiation, Inflammation, and Cell Cycle Control

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Summary

BCL-6, a transcriptional repressor frequently translocated in lymphomas, regulates germinal center B cell differentiation and inflammation. DNA microarray screening identified genes repressed by BCL-6, including many lymphocyte activation genes, suggesting that BCL-6 modulates B cell receptor signals. BCL-6 repression of two chemokine genes, MIP-1α and IP-10, may also attenuate inflammatory responses. Blimp-1, another BCL-6 target, is important for plasmacytic differentiation. Since BCL-6 expression is silenced in plasma cells, repression of *blimp-1* by BCL-6 may control plasmacytic differentiation. Indeed, inhibition of BCL-6 function initiated changes indicative of plasmacytic differentiation, including decreased expression of c-Myc and increased expression of the cell cycle inhibitor p27kip1. These data suggest that malignant transformation by BCL-6 involves inhibition of differentiation and enhanced proliferation.

Introduction

The transcriptional repressor BCL-6 has emerged as a multifunctional regulator of lymphocyte differentiation and immune responses (reviewed in Dalla-Favera et al., 1999; Staudt et al., 1999). BCL-6 mutant mice display two prominent phenotypes: a failure to form germinal centers during a T cell–dependent immune response and a fatal inflammatory disease distinguished by the presence of T helper type 2 (TH2) cells (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). Although the molecular mechanisms underlying these phenotypes are largely unknown, clues to the function of BCL-6 come from its expression pattern. Although *BCL-6* mRNA can be detected in many tissues (Allman et al., 1996), its protein expression is limited mainly to lymphocytes (Cattoretti et al., 1995; Onizuka et al., 1995; Allman et al., 1996), with the highest level of BCL-6 protein expressed in germinal center (GC) B cells. BCL-6 protein expression in B cells is exquisitely regulated following antigen encounter. Nascent pre-GC B cells upregulate BCL-6 protein, migrate to the follicular area, and initiate GC formation (Fukuda et al., 1997). In contrast, antigen-specific B cells that do not upregulate BCL-6 protein expression differentiate in the periarteriolar lymphoid sheath (PALS) into plasmablasts and provide an initial burst of low-affinity antibody (Fukuda et al., 1997). Whereas GC differentiation is blocked in BCL-6-deficient mice, plasmacytic differentiation within the PALS occurs normally (Fukuda et al., 1997). These observations suggest that BCL-6 controls a cell fate decision made by antigen-specific B cells, with high BCL-6 protein expression promoting GC differentiation and blocking plasmacytic differentiation within the PALS. BCL-6 may also regulate post-GC plasmacytic differentiation. While most GC B cells express BCL-6 protein, a minority do not and instead express MUM1/IRF-4, a gene highly expressed in plasma cells (Falini et al., 2000). These BCL-6-negative GC B cells include cells with plasmacytic morphology and may represent cells that are terminally differentiating as they exit the GC (Falini et al., 2000). Thus, plasmacytic differentiation, both pre- and post-GC, occurs only in the absence of BCL-6 expression.

Roughly one-sixth of all B cell non-Hodgkin's lymphomas have translocations of the *BCL-6* gene, making *BCL-6* one of the most frequently translocated genes in these cancers (reviewed in Dalla-Favera et al., 1999; Staudt et al., 1999). An attractive hypothesis is that *BCL-6* translocations cause non-Hodgkin's lymphomas by coopting BCL-6's regulatory functions during B cell differentiation. In keeping with this idea, the *BCL-6* translocations do not disrupt the *BCL-6* coding region but invariably substitute the BCL-6 promoter with a variety of other promoters. Thus, *BCL-6* translocations likely cause transformation of B cells by deregulating the expression of normal BCL-6 protein.

The aim of the present study was to discover the molecular pathways regulated by BCL-6 in order to understand how BCL-6 controls immune responses and promotes the formation of lymphomas. Although the consensus DNA binding site of BCL-6 has been defined (Baron et al., 1995; Seyfert et al., 1996), the genomic targets of BCL-6 repression remain largely unknown. The BCL-6 consensus binding site resembles the "GAS" motif recognized by the STAT family of transcription factors, raising speculation that BCL-6 may repress some cytokine response genes (Dent et al., 1997; Gupta et al., 1999; Harris et al., 1999). To comprehensively identify BCL-6 target genes, we used Lymphochip microarrays (Alizadeh et al., 1999, 2000). BCL-6 was found to repress a number of genes involved in B cell activation and terminal differentiation, inflammation, and cell cycle regulation, providing rich insights into the roles BCL-6 plays in the immune system and in human lymphomas.

Results

BCL-6 Expression Systems

To screen for BCL-6 target genes, we designed independent and complementary cellular systems in which BCL-6 function could be positively or negatively modulated. Gain-of-function systems were created by introducing full-length (FL) BCL-6 into cell lines lacking endogenous BCL-6 protein expression (Figure 1A). Loss-of-function systems were created by expressing a dominant-negative form of BCL-6, consisting solely of the BCL-6 zinc finger (ZnF) DNA binding domain, in cells that normally

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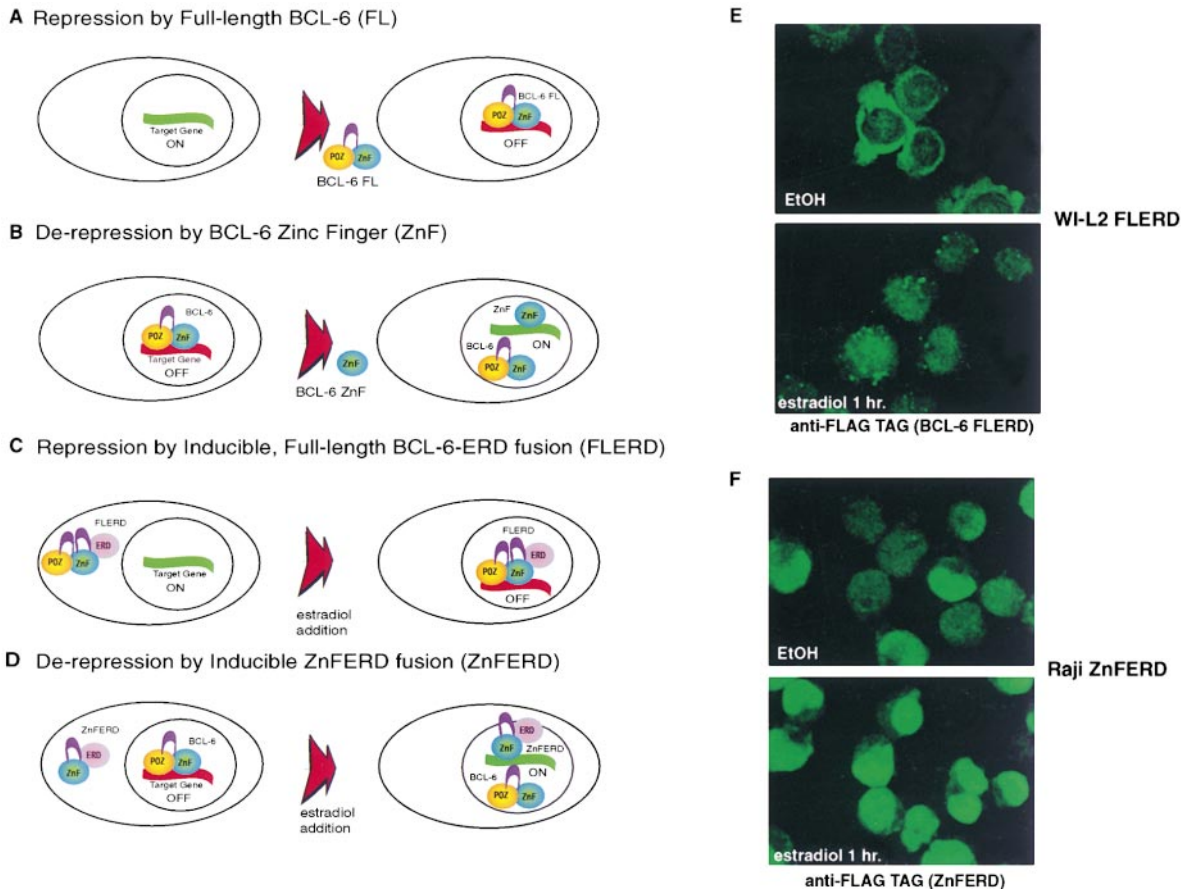

Figure 1. BCL-6 Expression Systems

(A–D) Cell systems in which BCL-6 activity was manipulated. See text for details.

(E–F) Detection of FLERD and ZnFERD proteins by immunofluoresence. WI-L2 cells carrying the FLERD vector (E) and Raji cells transduced with the ZnFERD virus (F) were treated with ethanol (noninducer) or estradiol (inducer) and then immunostained to detect the subcellular localization of these BCL-6-estrogen receptor fusion proteins.

express BCL-6 protein (Figure 1B). The BCL-6 ZnF domain does not repress transcription in transient transfection assays (Seyfert et al., 1996) and should thus block the ability of endogenous BCL-6 to repress transcription of its target genes. In most experiments, cell lines were transduced with bicistronic retroviruses expressing various forms of *BCL-6* together with a puromycin resistance gene, allowing rapid selection of polyclonal populations for analysis.

To more directly identify BCL-6 targets, we created two inducible forms of BCL-6 by fusing either full-length *BCL-6* or its ZnF domain to the ligand binding domain of the estrogen receptor (ERD; Figures 1C and 1D), allowing regulation of these proteins by estradiol. Typically, estradiol addition causes the translocation of transcription factor fusion proteins from the cytoplasm to the nucleus (Briegel et al., 1996). In some cases, the subcellular localization is less affected, and regulation presumably involves an alteration in the function of the transcription factor by the conformational changes in the ERD (Francis et al., 1995), which can itself act as a transcriptional activation domain (Cavailles et al., 1994). Since the inducible functions of ER fusion proteins do not require new protein synthesis, induction of these proteins can be conducted in the presence of protein synthesis inhibitors, which permits the identification of gene expression changes that are primary effects of the transcription factor. Repression by inducible BCL-6 is apparently due to its translocation from cytoplasm to nucleus upon binding estradiol, as demonstrated in a stable transfectant of the WI-L2 cell line (Figure 1E). The inducible, dominant-negative form of BCL-6 (ZnFERD) was found in both the cytoplasm and nucleus, but upon estradiol addition, ZnFERD moved quantitatively to the nucleus (Figure 1F). The ability of ZnFERD to block BCL-6 function may be due in part to its redistribution and in part to a conformational effect on its ability to interact with target genes and to act as an activator (via the ERD) of transcription.

Identification of BCL-6 Target Genes

Using the systems described above, we studied the effect of manipulating BCL-6 function on the expression of genes represented on Lymphochip microarrays (Alizadeh et al., 1999, 2000). Each experiment compared relative gene expression in two cell samples, typically cells retrovirally transduced with a form of *BCL-6* and cells transduced with a control retrovirus. Total RNA was used as a template to make cDNA probes, incorporating

either fluorescently labeled Cy3-dUTP (green) or Cy5-dUTP (red). Probes were mixed and hybridized to a single microarray. The hybridization of the two probes to each microarray spot was quantitated, and the fluorescence intensity ratio (Cy5/Cy3) was calculated as a relative measure of gene expression. For data analysis, a color scale was used to represent the ratios as colored boxes arranged in a matrix (Figure 2A; Alizadeh et al., 2000). Probe labeling for these experiments was designed such that genes that were repressed by introduction of BCL-6 into a BCL-6 null cell line, and genes that were induced by interference with BCL-6 in a BCL-6-positive cell line appear red in Figure 2A (i.e., Cy5/Cy3 $>$ 1).

In this initial screen, 13 arrays (representative of over 30 experiments) yielded ~80,000 individual measures of gene expression. The most likely BCL-6 target genes are those whose mRNA levels were changed in more than one cell type by manipulation of BCL-6 function. Figure 2A presents the 14 genes that met this criterion; expression levels of other genes were unchanged or altered in a cell line–specific manner and not included in this analysis (data not shown). These putative BCL-6 target genes were both repressed by the expression of full-length BCL-6 in BCL-6 null cells (K562, WI-L2, or SUDHL5) and induced (derepressed) by the expression of dominant-negative BCL-6 in cells expressing endogenous BCL-6 (Raji or BJAB). One previously proposed BCL-6 target gene, *CD23* (Dent et al., 1997; Gupta et al., 1999), was not identified in any of the present cell systems, whereas another potential BCL-6 target gene, *sterile* ε (Harris et al., 1999), was not included on the arrays used in this study.

To better identify which genes may be direct targets of BCL-6 repression, some experiments with inducible estrogen receptor fusion proteins were performed in the presence of the protein synthesis inhibitor cycloheximide. Most of the genes in Figure 2A, with the possible exception of *CD44*, *p27kip1*, and *Id2*, were altered in their expression despite inhibition of new protein synthesis, indicating that BCL-6 may directly bind to the regulatory regions of these genes and repress their transcription.

Some cell type–specific differences in response to BCL-6 manipulation are seen in Figure 2A. For instance, *CXCR4* does not appear as a target gene in K562 or WI-L2, while it is strongly repressed by BCL-6 in SUDHL5, a finding explained by the lack of CXCR4 expression in the former two cell lines and robust CXCR4 expression in the latter cell line (data not shown). In other cases, differences in regulatory factors in various cell types may be responsible for the variable effects of BCL-6 manipulation. For example, if a cell line lacks a positively acting transcription factor that is required for the expression of a given gene, derepression of BCL-6 in that cell line will have no effect. Finally, the expression of some genes may be at or below the detection threshold of microarrays and were therefore excluded from Figure 2A. For example, the hybridization signal for blimp-1 in SUDHL5 cells did not satisfy the data selection criteria (Figure 2A), but Northern blot analysis demonstrated that BCL-6 represses *blimp-1* in this cell type (see below).

Northern blot and semiquantitative RT-PCR analyses confirmed the ability of BCL-6 to alter transcription of the 14 target genes (Figure 3). There was close quantitative agreement between the microarray and Northern blot

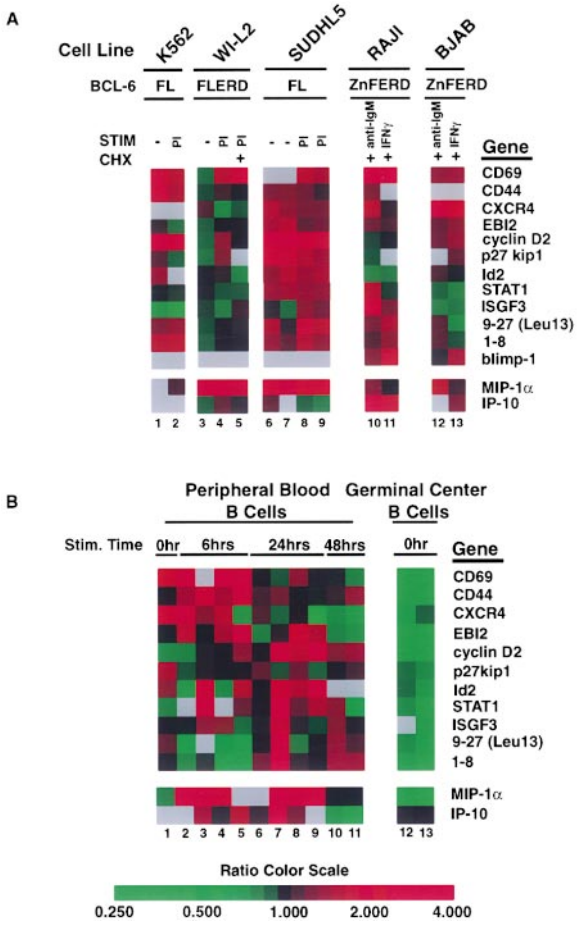

Figure 2. Microarray Screening for BCL-6 Target Genes

(A) Cell line microarray analysis. Each column is a single experiment comparing two cDNA populations, one labeled red (Cy5) and one labeled green (Cy3). Each row represents data from a single cDNA microarray spot. The red-to-green (Cy5/Cy3) ratio reflects hybridization to that spot, a measure of relative gene expression; intensity reflects the magnitude of the difference between the samples according the ratio color scale. Potential target genes repressed by BCL-6 (FL or FLERD, arrays 1–9) or derepressed by zinc finger (ZnFERD, arrays 10–13) have Cy5/Cy3 ratios $>$1 and are red. Green indicates Cy5/Cy3 ratios $<$1, black indicates no significant change in gene expression, and gray indicates the spot did not meet data selection criteria. The manner of stimulation for each cell line is indicated (STIM) as well as the preaddition of the translation-inhibitor cycloheximide (CHX).

(B) Expression of BCL-6 target genes in primary B cells. Microarray data were obtained from http://llmpp.nih.gov/lymphoma and derived from previous Lymphochip gene expression analyses of resting or mitogenically activated peripheral blood B cells (PBB) and germinal center B cells (GCB) (Alizadeh et al., 2000). Lane 1, PBB; lane 2, PBB α-IgM, 6 hr; lane 3, PBB α-IgM + CD40 ligand (CD40L), 6 hr; lane 4, PBB α-IgM + IL-4, 6 hr; lane 5, PBB α-IgM + CD40L + IL-4, 6 hr; lane 6, PBB α-IgM, 24 hr; lane 7, PBB α-IgM + CD40L, 24 hr; lane 8, PBB α-IgM + IL-4, 24 hr; lane 9, PBB α-IgM + CD40L + IL-4, 24 hr; lane 10, PBB α-IgM + CD40L, 48 hr; lane 11, PBB α-IgM + CD40L, 48 hr; lane12, GCB; and lane 13, GCB.

analysis (see http://lymphochip.nih.gov/shafferetal/). Of particular note was the fact that *blimp-1* was repressed by BCL-6 in SUDHL5 cells (Figure 3B) and induced by two dominant-negative BCL-6 proteins (ZnFERD [Figure 3B] and ZnF [Figure 3C]) in Raji cells. Interestingly,

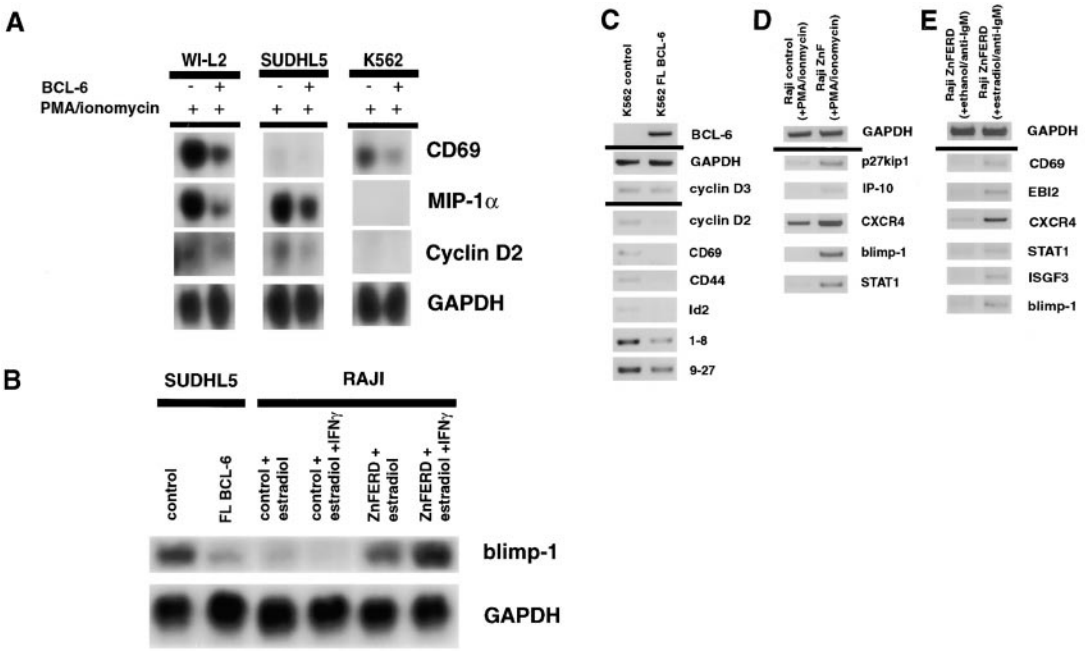

Figure 3. Northern Blot and RT-PCR Confirmation of BCL-6 Target Genes

(A) Northern blot analysis confirms BCL-6 repression of target genes. Cell line RNA was probed with fragments of the coding region for each gene. A GAPDH probe was used as a control. The presence of active BCL-6 is indicated: SUDHL5 and K562, transduced with FL BCL-6 virus (plus) or control virus (minus); WI-L2, transfected with FLERD, treated with estradiol (plus) or with EtOH (minus). Treatment with PMA and ionomycin is indicated (plus).

(B) Northern blots for *blimp-1* expression were performed as in (A) on RNA from SUDHL5 and Raji (± estradiol pretreatment, followed by interferon-γ addition as indicated).

(C) RT-PCR analysis of target genes in K562 cells transduced with control or FL BCL-6 virus shows BCL-6 repression of target genes.

(D) RT-PCR analysis shows induction of target genes in Raji cells transduced with ZnF virus as compared to control cells after treatment with PMA and ionomycin.

(E) RT-PCR analysis of Raji cells transduced with ZnFERD virus and stimulated by anti-IgM cross-linking shows induction of target genes in the presence of the inducer (estradiol) but not the noninducer (EtOH). *GAPDH* and *cyclin D3* amplification shows the equivalence of the mRNA amplification and loading.

blimp-1 could be further induced by interferon-γ treatment of Raji cells only when ZnFERD was activated by estradiol (Figure 3B). These results suggest that endogenous BCL-6 in Raji cells represses basal blimp-1 expression and makes the *blimp-1* gene insensitive to transcriptional activation by other transcription factors, including the STAT factors.

Expression of BCL-6 Target Genes During B Cell Activation and Differentiation

Several of the BCL-6 target genes discovered in this analysis are induced during activation of mature B cells including *CD69*, *CD44*, *cyclin D2*, and *MIP-1α* (Camp et al., 1991; Lopez-Cabrera et al., 1995; Solvason et al., 1996; Krzysiek et al., 1999). We predicted that most BCL-6 target genes would be expressed at higher levels in activated peripheral blood B cells than in GC B cells, since BCL-6 protein is not expressed in resting or activated B cells but is strongly expressed in most GC B cells (Cattoretti et al., 1995; Onizuka et al., 1995; Allman et al., 1996). A database of gene expression from normal human B cells (Alizadeh et al., 2000; http://llmpp.nih.gov/lymphoma) was queried for the expression of each of the BCL-6 target genes in resting peripheral blood B cells; in peripheral blood B cells activated in vitro with various combinations of anti-IgM, CD40 ligand, and IL-4; and in GC B cells (Figure 2B). Many of the BCL-6 target genes are activation genes, induced 6–24 hr following stimulation. As predicted, most BCL-6 target genes were expressed at much lower levels in GC B cells (Figure 2B, lanes 12 and 13). Blimp-1, known to be preferentially expressed in plasma cells, was not expressed detectably above background in any of the B cell samples in Figure 2B (data not shown). These results suggest that elevated BCL-6 protein in GC B cells represses these target genes and that low levels of BCL-6 in mitogenically activated peripheral blood B cells (Allman et al., 1996) allow these activation genes to be expressed.

Repression of Target Gene Promoters by BCL-6

The regulatory regions of several target genes were scanned for the consensus BCL-6 binding motif, 5′-YTC CTAGAR-3′ (Seyfert et al., 1996). The human *cyclin D2*, *MIP-1α*, and *CD69* genes were each found to have at least one potential BCL-6 binding site. Remarkably, the BCL-6 site in the human *cyclin D2* promoter is conserved in both the mouse and rat *cyclin D2* genes (Jun et al., 1997; Yang et al., 1997), and the BCL-6 site in the human *MIP-1α* promoter is conserved in the mouse *MIP-1α* gene (Widmer et al., 1993). An electrophoretic mobility shift DNA binding assay showed that BCL-6 protein was able to bind specifically to the *cyclin D2*, *MIP-1α*, and *CD69* sites (see http://lymphochip.nih.gov/shafferetal/).

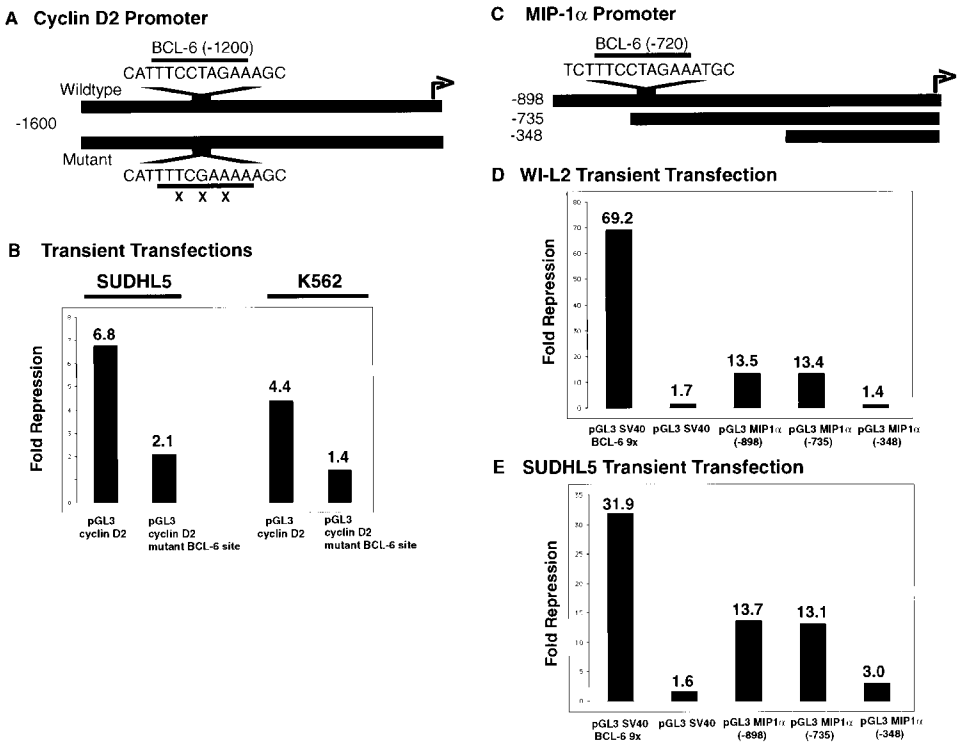

Figure 4. Regulation of Target Gene Promoters by BCL-6

Transient transfection of luciferase reporters into BCL-6-negative target cell lines, with and without BCL-6 cotransfection, was performed. Fold-repression of transcription by BCL-6, as measured by luciferase activity, was calculated by dividing luciferase activity in the absence of BCL-6 by the activity in the presence of BCL-6 (normalized for transfection efficiency using a β-gal reporter). Control constructs consisted of the SV40 promoter with nine tandem BCL-6 sites cloned upstream (pGL3 SV40 9x BCL-6, positive repression control) and the SV40 promoter alone (pGL3 SV40, no BCL-6 sites) as a control for site-dependence of repression.

(A) Schematic of the human *cyclin D2* promoters (wild type and mutant) with mutations disrupting the BCL-6 site indicated (X).

(B) Transient transfection shows BCL-6 repression of the human wild-type *cyclin D2* promoter in SUDHL5 and K562 compared to the mutant promoter.

(C) Schematic of the human *MIP-1α* promoter deletion constructs, with the BCL-6 site indicated.

(D and E) Transient transfection shows that BCL-6 repression of the human *MIP-1α* promoter in WI-L2 (D) and SUDHL5 (E) depends upon the presence of the BCL-6 site.

To directly assess the ability of BCL-6 to repress transcription through these sites, we generated constructs in which the human *MIP-1α* and *cyclin D2* promoters drive expression of a luciferase reporter gene (Figures 4A and 4C). The activity of these reporters was measured in the presence or absence of cotransfected BCL-6 expression vectors. A series of *MIP-1α* promoter constructs showed that BCL-6 repressed promoter activity of those constructs with a BCL-6 binding site (~13-fold) in both WI-L2 and SUDHL5 cells but had much less effect on the activity of the construct lacking the BCL-6 binding site (Figures 4D and 4E). Likewise, BCL-6 repressed the activity of the wild-type *cyclin D2* promoter 4- to 6.8-fold in SUDHL5 and K562 cells, and this repression was substantially reduced when the BCL-6 binding site was mutated (Figure 4B). These data establish both *MIP-1α* and *cyclin D2* as direct targets of BCL-6 repression acting through specific binding sites in their promoters.

BCL-6 Alters Basal and Induced Levels of Target Gene Proteins

Since the candidate BCL-6 target genes *CD44*, *Leu13*, *CD69*, and *CXCR4* encode membrane proteins, we used flow cytometry to monitor their expression in response to alterations in BCL-6 activity (Figure 5). Both SUDHL5 and K562 express high constitutive levels of CD44 protein, and the expression of BCL-6 decreased cell surface expression of CD44 substantially (Figure 5A). Similarly, BCL-6 also decreased CD69 protein expression in SUDHL5 cells (Figure 5D). In Raji cells, blocking BCL-6 function resulted in elevated surface expression of Leu13 (Figure 5B), CD69 (Figure 5C), and CXCR4 (Figure 5E), suggesting that these genes are repressed by the endogenous BCL-6 protein in Raji cells.

We next asked whether manipulating BCL-6 function could alter induced expression of target genes. Expression of BCL-6 in SUDHL5 substantially inhibited the ability of PMA plus ionomycin to increase CD69 expression (Figure 5D). In Raji cells, PMA plus ionomycin alone did not induce CXCR4 (Figure 5E), while in cells with dominant-negative BCL-6, blocking BCL-6 increased the basal expression of CXCR4 and promoted an additional increase in expression upon stimulation (Figure 5E). These observations suggest that the endogenous BCL-6 protein in Raji cells blocks both constitutive and induced expression of CXCR4.

Since PMA plus ionomycin is a pharmacological

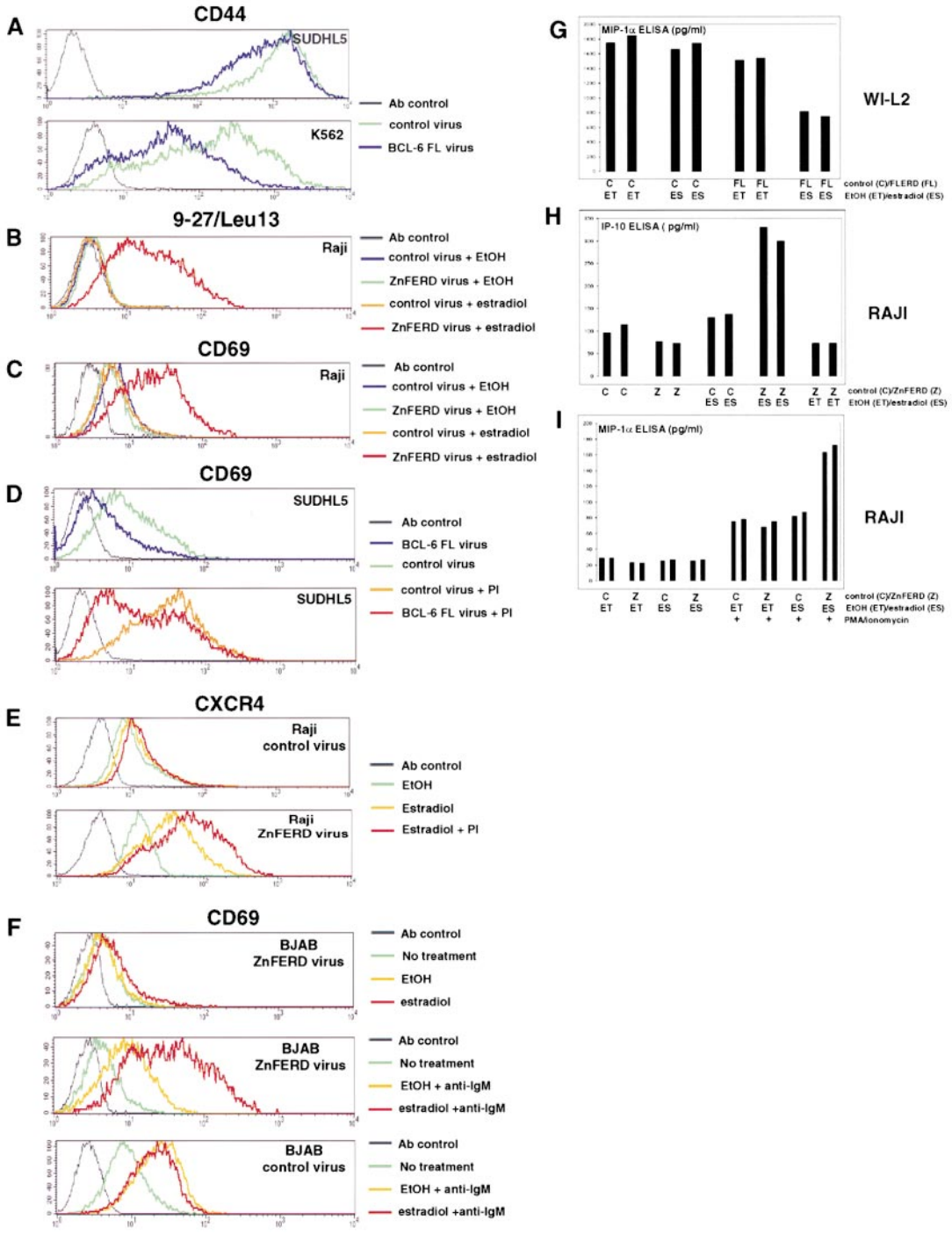

Figure 5. Regulation of Surface Marker and Chemokine Expression by BCL-6

(A) Repression of CD44 by BCL-6 in SUDHL5 and K562. The green trace shows control cells; the blue trace represents BCL-6-virus transduced cells. The black line shows staining with an isotype-matched, nonspecific control. Data are representative of two or more independent infections.

(B) Derepression of 9–27/LEU13 by ZnFERD in Raji.

(C) Derepression of CD69 by ZnFERD in Raji.

(D) Repression of basal and inducible (± PMA and ionomycin) CD69 by BCL-6 in SUDHL5.

(E) Derepression of CXCR4 by ZnFERD in Raji.

(F) Derepression and superinduction of CD69 by ZnFERD upon BCR cross-linking in BJAB. Cells were stained for CD69 in the absence (top panel) or presence (lower panels) of anti-IgM. Cells were treated with ethanol or estradiol prior to addition of anti-IgM.

(G) Inducible MIP-1α expression is blocked by BCL-6. MIP-1α levels were measured in WI-L2 control cells (c) and WI-L2 FLERD cells (FL) after treatment with EtOH (ET) or estradiol (ES).

mimic of signaling through the B cell antigen receptor (BCR), we tested whether the activation of gene expression by anti-IgM cross-linking of the BCR could also be augmented by blocking BCL-6 function. The EBV-negative Burkitt's lymphoma cell line BJAB has been studied as a model for signaling through the B cell antigen receptor (Miller et al., 1993). In unstimulated BJAB cells transduced with dominant-negative BCL-6, estradiol treatment had no effect on surface CD69 expression (Figure 5F), in contrast to Raji cells (Figure 5C). When BJAB cells were stimulated by BCR cross-linking, CD69 expression was increased, and this effect was greatly enhanced by blocking BCL-6 (Figure 5F). In BJAB cells transduced with control virus, estradiol treatment did not superinduce CD69 expression following anti-IgM stimulation. These findings suggest that BCL-6 interferes with the activation of genes in response to signals through the BCR.

The chemokines *MIP-1*α and *IP-10* form an important class of BCL-6 target genes given that one of the prominent phenotypes of BCL-6 mutant mice is a fatal inflammatory disease (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). To confirm the results of the microarray analysis, we measured the secretion of these chemokines by ELISA under conditions in which BCL-6 activity was altered. BCL-6 expression in WI-L2 cells inhibited the constitutive secretion of MIP-1α (Figure 5G). Induction of dominant-negative BCL-6 in Raji cells increased secretion of IP-10, suggesting that endogenous BCL-6 protein represses the basal expression of the *IP-10* gene (Figure 5H). In the same cell system, blocking BCL-6 alone did not increase the basal secretion of MIP-1α (Figure 5I). Interestingly, blocking BCL-6 did enhance the induction of MIP-1α following PMA plus ionomycin treatment (Figure 5I). These results suggest that the endogenous BCL-6 protein in Raji cells represses activated transcription of the *MIP-1*α gene. Further, these data support a role for BCL-6 in the inhibition of chemokine secretion by B cells.

BCL-6 Target Genes in BCL-6 Mutant Mice

Given the ability of BCL-6 to repress genes involved in B cell activation and differentiation in a variety of in vitro systems, we next analyzed B cells from BCL-6 mutant mice (Dent et al., 1997). The expression of several BCL-6 target genes in splenic B cells from BCL-6 heterozygous control ($+/-$) and BCL-6 mutant ($-/-$) littermates was analyzed by flow cytometry (Figure 6A). BCL-6 mutant mice were found to have B cells with an activated cell surface phenotype. First, when stained for expression of the pan-B cell marker B220 and for surface IgM expression, B cells in BCL-6 mutant mice expressed 10-fold lower levels of surface IgM than control littermates (Figure 6A). This decrease in IgM staining is similar to that seen 2 days after immunization of control mice with sheep red blood cells (Figure 6A). B cell activation is accompanied by a decrease in surface IgM expression (Wheeler and Gordon, 1996), implying that BCL-6 mutant B cells may be spontaneously activated in the absence of deliberate immunization. Second, splenic B cells from control mice showed a range of CD44 expression over 2 orders of magnitude (Figure 6B). In contrast, B cells from the BCL-6 mutant mice had uniformly high CD44 expression, a phenotype associated with B cell activation (Camp et al., 1991). Lastly, very few ($\sim$2%) of the B cells from unimmunized heterozygous mice expressed the lymphocyte activation marker CD69, and immunization modestly increased the proportion of $CD69^+$ B cells to 8% (Figure 6C). In sharp contrast, 55% of the B cells in BCL-6 mutant mice expressed CD69. This "activated" B cell phenotype ($IgM^{low}CD44^{high}CD69^+$) was observed in multiple BCL-6 mutant mice from independent litters and had no apparent correlation with the health or age of the BCL-6 mutant mice.

Given that BCL-6 mutant mice have an influx of inflammatory cells in the spleen when immunized (Dent et al., 1997), we tested whether secretion of the chemokine and inflammatory mediator MIP-1α is elevated in these mice. MIP-1α is induced and secreted by normal B cells following mitogenic activation (Krzysiek et al., 1999). We therefore compared MIP-1α secretion by control and BCL-6 mutant splenic B cells following in vitro activation with lipopolysaccaride (LPS). BCL-6 mutant B cells secreted 6.4-fold more MIP-1α after 3 days of culture with LPS than did heterozygous B cells (Figure 6D). This result raises the possibility that the splenic inflammation that accompanies immunization of BCL-6 mutant mice may be due, in part, to the elevated secretion of MIP-1α by B cells in the absence of BCL-6. The analysis of BCL-6 mutant mice provides complementary genetic evidence that *CD69*, *CD44*, and *MIP-1*α are indeed targets of BCL-6 repression.

Dominant-Negative BCL-6 Alters Differentiation Markers and Arrests Growth

Earlier studies of Raji cells transduced with dominant-negative BCL-6 were performed in the presence of the protein synthesis inhibitor cycloheximide in order to identify direct BCL-6 target genes (Figure 2A). To investigate the downstream effects of blocking BCL-6 function in the Raji cells, we repeated these experiments in the absence of cycloheximide, and monitored gene expression using Lymphochip microarrays at 3, 8, 24, and 48 hr after estradiol induction of ZnFERD. Inhibition of BCL-6 function in Raji cells altered the expression of cell cycle–regulated genes, B cell differentiation genes, interferon-responsive genes, and the previously identified BCL-6 target genes (Figure 7A; data not shown). A notable member of the cell cycle–regulated class was c-*myc*, a gene that is repressed by blimp-1 (Lin et al., 1997). The fact that *blimp-1* is induced in Raji cells when BCL-6 is blocked (Figures 3B and 7A) suggests that the decreased c-*myc* expression is secondary to increased *blimp-1* expression. c-Myc protein was correspondingly decreased under the same conditions (Figure 7B). Interestingly, two genes that are activated by c-Myc, *lactate dehydrogenase* (Shim et al., 1997) and *ornithine decarboxylase* (Wagner et al., 1993), were also lowered in expression with slightly delayed kinetics compared with c-*myc* (Figure 7A). A number of other cell cycle–related

(H) ZnFERD derepresses IP-10 production in Raji. Control (c) and ZnFERD (z) transduced pools were left untreated or treated with ethanol (ET) or estradiol (ES).

(I) ZnFERD derepresses inducible MIP-1α production in Raji. Raji control (c) and ZnFERD (z) transduced pools were treated with ethanol (ET) or estradiol (ES) alone or followed by PMA and ionomycin (plus). Secreted chemokine levels were determined in duplicate.

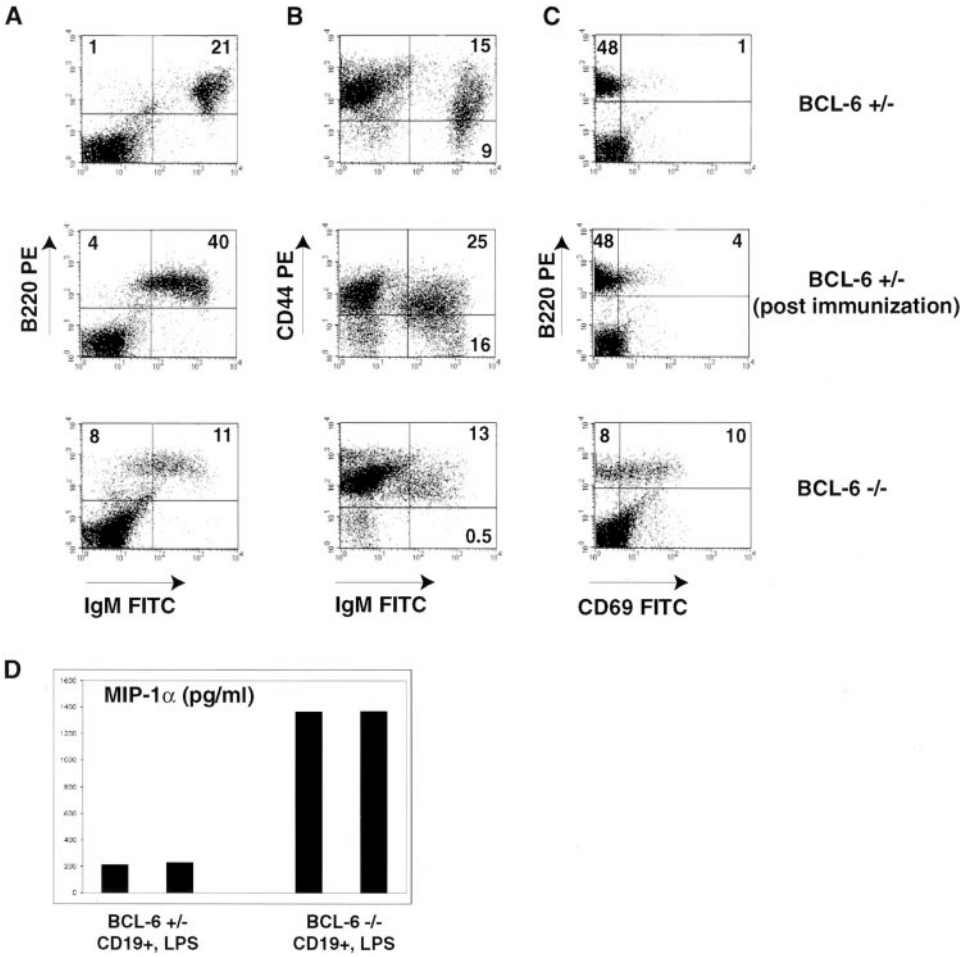

Figure 6. Inappropriate B Cell Activation and Chemokine Secretion in BCL-6 Mutant Mice

(A–C) Flow cytometry of B cells from BCL-6 mutant (−/−) and control (+/−) littermates. BCL-6 (+/−) mice were also injected intraperitineally (i.p.) with sheep red blood cells (srbc) two days prior to sacrifice (middle row). (A) Staining for the pan-B cell marker B220 (PE) and for surface IgM (FITC). Percentages of $B220^{+}$, IgM^{hi}, and IgM^{lo} cells are indicated in each panel. (B) Staining for IgM (FITC) and CD44 (PE). Percentages of IgM^{+}, $CD44^{hi}$, and $CD44^{lo}$ cells are indicated in each panel. (C) Staining for B cells (B220, PE) and CD69 (FITC). Percentages of $B220^{+}$, CD69-positive and -negative B cells are indicated in each panel.

(D) B cells overexpress MIP-1α in the absence of BCL-6. Equal numbers of purified ($CD19^{+}$) BCL-6 heterozygous (+/−) and BCL-6 mutant (−/−) B cells were cultured for 3 days with LPS. Supernatants were analyzed in duplicate by ELISA for secreted MIP-1α. Data from one of two independent B cell purification/stimulation experiments are shown.

genes were also downregulated by blocking BCL-6, while several growth arrest genes, including *p27kip1*, were induced under these conditions (Figure 7A).

Profound changes in cell cycle genes induced by blocking BCL-6 function correlated with changes in cell viability and cell cycle distribution. Cultures of ZnFERD-transduced Raji cells treated with estradiol had fewer viable cells and more dead cells than similarly treated control Raji cells (Figure 7D). Further, induction of ZnFERD arrested the cell cycle, as evidenced by accumulation of cells in G1 phase and loss of cells in S and G2/M phases (Figure 7E).

A number of B cell differentiation genes were altered in expression by interfering with BCL-6 function in Raji cells, and many of these gene expression changes mimicked those that accompany normal plasma cell differentiation. Several genes that are characteristically expressed in GC cells were downregulated when BCL-6 function was blocked (*CD10*, *A-myb*, *CD27*, *CD70*, and *BCL-7A*; Alizadeh et al., 2000) as were a number of pan-B cell genes (*CD19*, *CD37*, *CD79A*, *CD79B*, *Spi-B*, and *CD20*) (Figures 7A and 7C). The expression of many of these genes is also downregulated in plasma cells (Liu and Banchereau, 1996). Conversely, genes that are upregulated during terminal plasmacytic differentiation were induced (*CD38*, *blimp-1*, and *MCL-1*; Turner et al., 1994; Liu and Banchereau, 1996) (Figures 7A and 7C). However, this apparent plasmacytic differentiation was incomplete as these cells failed to express the plasma cell marker syndecan-1 and also upregulated *MHC class II*, genes that are not typically expressed by plasma cells.

Discussion

Using cDNA microarrays in a gene expression screen, we identified genes that are potential targets of BCL-6

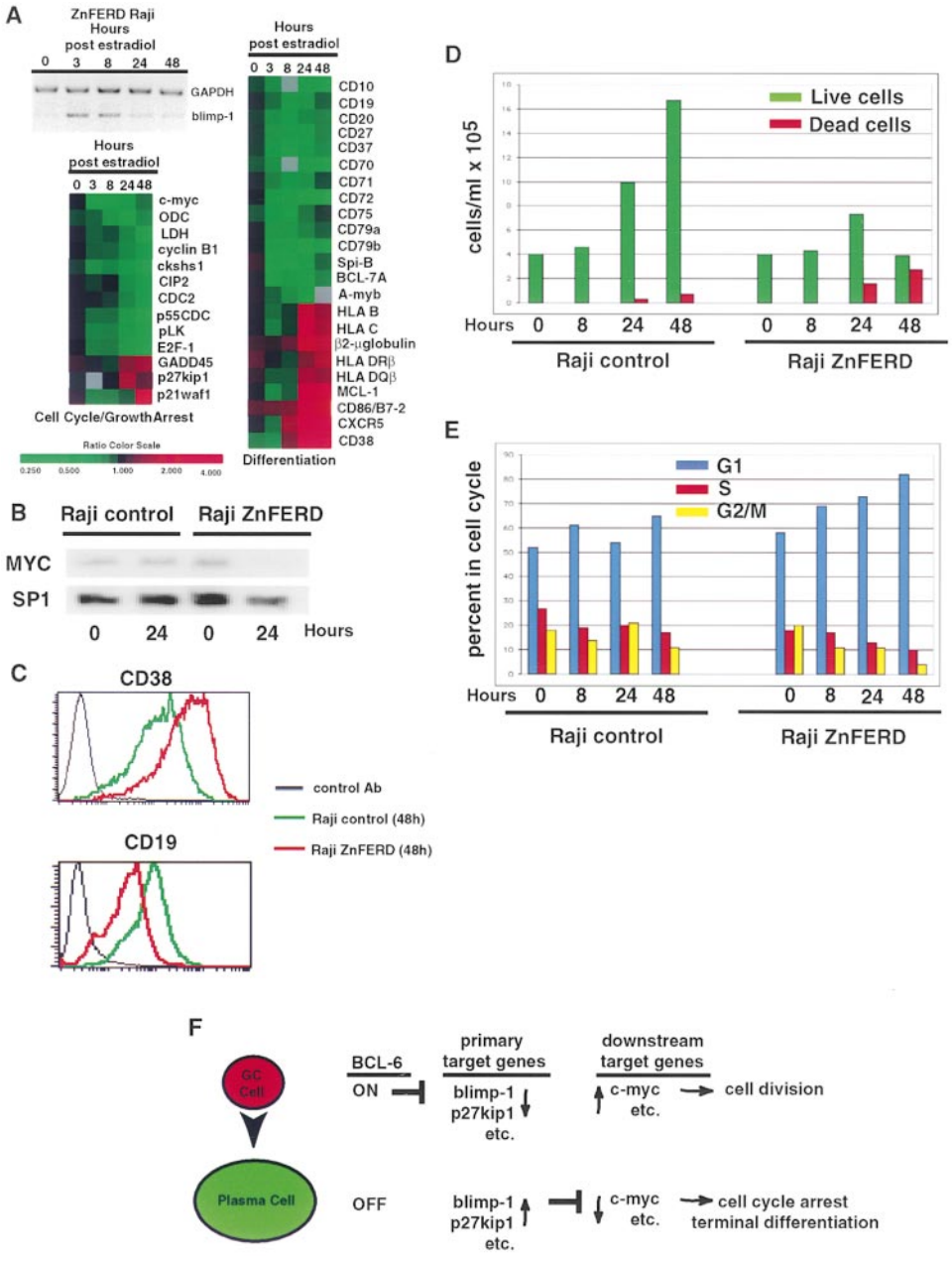

Figure 7. Inhibition of BCL-6 Alters Differentiation Markers and Arrests Cell Division

(A) Gene expression changes following estradiol induction of control versus ZnFERD-transduced Raji cells. RNA was harvested before induction (time 0) and at 3, 8, 24, and 48 hr after estradiol addition. RT-PCR for *blimp-1* shows its induction in ZnFERD virus-transduced cells, while control cells do not express blimp-1 (data not shown). Also shown are microarray data comparing control cells to ZnFERD-transduced cells. Genes upregulated following blockade of BCL-6 function by ZnFERD are depicted in red; downregulated genes are depicted in green.

(B) Western blot analysis shows c-Myc downregulation in ZnFERD-induced cells compared to controls. The SP1 blot controls for quality and loading lysates.

(C) Differentiation markers are altered by BCL-6 inhibition. Flow cytometric analysis for CD38 and CD19 were performed at 48 hr after estradiol addition.

(D) Cell death increases when BCL-6 is inhibited.

(E) Cell cycle arrest follows BCL-6 inhibition. Control and ZnFERD-transduced cells were monitored for DNA content, as a measure of their position in the cell cycle, over the course of estradiol induction.

(F) Hypothesis: the role of BCL-6 in B cell differentiation. Germinal center B cells express BCL-6, which represses genes such as *p27kip1* and *blimp-1*, allowing other genes such as c-*myc* to promote rapid cell division while forestalling terminal differentiation. Terminal differentiation to plasma cells occurs when BCL-6 expression terminates and target genes like *blimp-1* initiate a new genetic program.

repression. These target genes are functionally linked by their roles in B cell activation (*CD69*, *CD44*, *EBI2*, *Id2*, *STAT1*), B cell differentiation (*blimp-1*), inflammation (*MIP-1α*, *IP-10*), and cell cycle control (*p27kip1*, *cyclin D2*). Multiple independent and complementary lines of evidence support the conclusion that these genes are BCL-6 targets. First, microarray data, along with confirmatory Northern blot and RT-PCR analysis, showed that these genes were repressed by ectopic expression of BCL-6 in BCL-6-negative cell lines and were activated by the expression of dominant-negative forms of BCL-6 in BCL-6-positive cell lines. Second, the mRNA levels of most target genes, with the possible exception of *CD44*, *p27kip1*, and *Id2*, could be altered by BCL-6 or its dominant-negative form in the presence of the protein synthesis inhibitor cycloheximide. This implies that these genes are direct targets of BCL-6 repression. Third, the promoter regions of three target genes, *MIP-1α*, *cyclin D2*, and *CD69*, were found to contain high-affinity BCL-6 binding sites. The BCL-6 binding sites in the *MIP-1α* and *cyclin D2* promoters have been highly conserved in evolution, and the promoters of these genes were repressed by the expression of BCL-6 in transient transfection assays. Fourth, the protein products of the CD44, Leu13, CD69, CXCR4, MIP-1α, and IP-10 genes were correspondingly modulated by alteration of BCL-6 function. Finally, genetic disruption of *BCL-6* in mice caused increased expression of three BCL-6 target genes in B lymphocytes: *CD69*, *CD44*, and *MIP-1α*. The functions of these potential BCL-6 target genes shed considerable light on the molecular mechanisms by which BCL-6 regulates GC formation and inflammation and causes lymphoma when dysregulated.

Further work will be required to define the precise molecular mechanisms by which BCL-6 alters the mRNA levels of each of these candidate target genes. Some target genes, such as *MIP-1α*, *cyclin D2*, and *CD69*, may be regulated by direct binding of BCL-6 to *cis*-elements in their promoters. BCL-6 can also interact with other transcription factors (Davies et al., 1999; Gupta et al., 1999), and thus it is possible that some of the target genes defined here result from interference or synergism between BCL-6 and these other factors.

Regulation of B Cell Differentiation by BCL-6

Many of the identified target genes are regulated in their expression during B cell activation and differentiation and thus are attractive candidates for BCL-6 regulation. All of the BCL-6 target genes were found to be expressed at low or undetectable levels in GC B cells and were expressed in resting and/or in vitro activated human peripheral blood B cells. This observation is consistent with the 3- to 30-fold higher expression of BCL-6 protein in GC B cells compared with resting peripheral blood B cells and with the downregulation of BCL-6 expression during mitogenic activation of peripheral blood B cells (Allman et al., 1996). Thus, upregulation of BCL-6 protein expression in GC B cells contributes to the characteristic gene expression signature of this stage of differentiation (Alizadeh et al., 2000).

An important theme uniting many of the BCL-6 target genes is induction via signals through the BCR. Several genes (*CD69*, *CD44*, *cyclin D2*, *STAT1*, *MIP-1α*, and *IP-10*) were shown to be B cell activation genes by microarray analysis (Alizadeh et al., 2000) and by more conventional assays. Indeed, in some experimental systems, BCL-6 had quantitatively greater effect on the induced expression of genes than on their basal expression. For example, repression of CD69 in SUDHL5 cells by BCL-6 was more pronounced after stimulation with PMA plus ionomycin, a pharmacological mimic of BCR signaling. Similarly, expression of dominant-negative BCL-6 in Raji cells caused a greater increase in CXCR4 expression in cells stimulated with PMA plus ionomycin. Perhaps the clearest demonstration of this principle was the effect of dominant-negative BCL-6 on CD69 expression in BJAB cells. Dominant-negative BCL-6 had no effect on basal CD69 levels in BJAB cells but led to augmented induction of CD69 following stimulation through the BCR. Since BCL-6 blocks expression of genes induced when B cells are stimulated by antigen, the outcome of signaling through the BCR in a GC B cell could be altered by the expression of BCL-6.

Blimp-1 is a transcriptional repressor which plays a key role in differentiation of B cells to plasma cells (Turner et al., 1994). Upregulation of *blimp-1* mRNA is an early event in the differentiation of mouse splenic B cells into antibody-secreting preplasma cells (Soro et al., 1999), and enforced expression of blimp-1 is sufficient to direct cells toward plasma cell differentiation (Turner et al., 1994; Messika et al., 1998). Blimp-1 represses the transcription of c-Myc (Lin et al., 1997; Knodel et al., 1999) and in B cells that are poised to differentiate to plasma cells, this drop in c-*myc* expression is integral to the process of terminal differentiation (Lin et al., 1997; Messika et al., 1998). Therefore, the repression of *blimp-1* by BCL-6 could be a pivotal event controlling the differentiation of GC B cells to plasma cells.

We observed that blockade of BCL-6 function in Raji cells not only induced *blimp-1* expression but also decreased c-*myc* expression and arrested the cell cycle in G1. Since c-Myc regulates the transition from G1 to S phase (Carman et al., 1996), the decrease in c-*myc* expression may be responsible, in part, for the observed cell cycle arrest. These results are consistent with a model in which BCL-6 inhibits *blimp-1*, which in turn inhibits c-*myc* (Figure 7F). Thus, elevated levels of BCL-6 in GC B cells would result in higher levels of c-Myc, whereas loss of BCL-6 in plasma cells would result in lower levels of c-Myc.

Expression of several other B cell differentiation genes was altered by inhibition of BCL-6 function, many of which are reminiscent of normal plasmacytic differentiation. A number of germinal center–restricted genes (Alizadeh et al., 2000) were downregulated (*CD10*, *A-myb*, and *BCL-7A*) as were CD19 and CD20, markers that are known to decrease during plasmacytic differentiation. Conversely, CD38 was upregulated as also occurs in plasma cells (Liu and Banchereau, 1996). Furthermore, blockade of BCL-6 caused cell cycle arrest, reminiscent of the arrest accompanying terminal plasma cell differentiation. However, some plasma cell markers such as syndecan-1 were not induced in Raji cells under these conditions, indicating that BCL-6 inhibition causes only partial plasmacytic differentiation in this cell system.

Taken together, these findings suggest that BCL-6 is a key upstream gatekeeper of terminal B cell differentiation (Figure 7F). Consistent with this model is the observation that unimmunized BCL-6 mutant mice have elevated numbers of IgG- and IgE-expressing plasmacytoid cells (Ye et al., 1997). BCL-6 may block plasmacytic differentiation at two distinct points during a normal antigen response. In the initial days after immunization,

a few BCL-6-expressing, pre-GC B cells appear in the PALS, whereas B cells undergoing plasmacytic differentiation in the PALS do not express BCL-6 (Fukuda et al., 1997). Thus, the elevated expression of BCL-6 in some B cells early in the antigen response may skew these B cells toward a germinal center fate and away from a plasmacytic fate. This model further suggests that the continued expression of BCL-6 in most GC B cells is required to repress *blimp-1* and block terminal plasma cell differentiation. Interestingly, while most B cells in the germinal center express BCL-6 protein, a minority of GC cells with either centrocytic or plasmacytic morphology do not and instead express MUM1/IRF-4, a transcription factor that is highly expressed in plasmacytic cells (Falini et al., 2000). Given the repression of blimp-1 by BCL-6, downregulation of BCL-6 may be a required first step in the decision of a GC B cell to initiate plasmacytic differentiation.

Regulation of Inflammatory Responses by BCL-6

A prominent and fatal phenotype of BCL-6 mutant mice is an inflammatory myocarditis and pulmonary vasculitis that develops shortly after birth. This inflammation is characterized by a mixture of lymphoid and myeloid cell types and has the hallmarks of a Th2 process (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). The observation that BCL-6 represses the chemokines MIP-1α and IP-10 suggests additional pathogenic mechanisms that may contribute to the inflammatory responses of BCL-6 mutant mice. MIP-1α, a CC chemokine, recruits inflammatory cells, including lymphocytes, monocytes, eosinophils, and mast cells, to sites of immune challenge (Schall et al., 1993; Lukacs et al., 1995). IP-10, a CXC chemokine, was originally identified as a chemoattractant specific for activated T cells but can also recruit B cells, monocytes, and NK cells during immune responses (reviewed in Farber, 1997). Both MIP-1α and IP-10 are induced by BCR stimulation, and thus an important role for BCL-6 may be to blunt the expression of these chemokines by B cells during an immune response. Dysregulated secretion of MIP-1α and/or IP-10 may therefore contribute to the splenic inflammation that occurs in BCL-6 mutant mice following immunization (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). Interestingly, MIP-1α has been shown to play a role in experimental models of myocarditis and pulmonary inflammation, and this inflammatory response does not occur in mice that are genetically deficient in MIP-1α expression (Cook et al., 1995). Further experiments will be needed to test directly whether abnormal expression of MIP-1α, or other chemokines, contributes to inflammatory disease in BCL-6-deficient mice.

Implications for Lymphomagenesis

The translocations of *BCL-6* in non-Hodgkin's lymphomas alter the *BCL-6* locus by substituting strong, constitutively active promoters for the *BCL-6* promoter (reviewed in Dalla-Favera et al., 1999; Staudt et al., 1999). These translocations most likely prevent the downregulation of *BCL-6* transcription that occurs upon plasmacytic differentiation. Lymphomas with *BCL-6* translocations would therefore maintain repression of BCL-6 target genes, trapping these cells at the germinal center stage of differentiation. Repression of *blimp-1* by BCL-6 might be especially critical for malignant transformation. As a transcriptional repressor of c-*myc*, blimp-1 can cause either growth arrest and terminal plasmacytic differentiation or apoptosis. Because BCL-6 represses *blimp-1*, which in turn represses c-*myc*, BCL-6 translocations might be considered functionally equivalent to c-*myc* translocations. In this regard, it is notable that Burkitt's lymphomas, which invariably have c-*myc* translocations, are one of the few non-Hodgkin's lymphoma subtypes that almost never have *BCL-6* translocations (reviewed in Dalla-Favera et al., 1999; Staudt et al., 1999). Conversely, non-Hodgkin's lymphomas with *BCL-6* translocations do not often carry c-*myc* translocations (Mitelman, 1994).

The repression of *p27kip1* by BCL-6 might also be critical for malignant transformation by BCL-6. p27kip1 arrests the cell cycle in response to extrinsic signals at the G1-to-S phase transition by binding to G1 cyclin-dependent kinases (Polyak et al., 1994; Toyoshima and Hunter, 1994). Mice deficient in p27kip1 develop multiorgan hyperplasia and tumors, emphasizing its critical role as a tumor suppressor (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Even heterozygous p27kip1 mutant mice develop tumors more readily than wild-type mice, and many human tumors have hemizygous loss of one allele of *p27kip1* (Fero et al., 1998). Small changes in p27kip1 levels can therefore affect malignant transformation. Germinal center centroblasts have one of the most rapid doubling times of any primary human cell type ($\sim$6 hr) and express no detectable p27kip1 (Quintanilla-Martinez et al., 1998; Sanchez-Beato et al., 1999). Therefore, BCL-6 repression of *p27kip1* may permit rapid cell cycle transit in both normal GC B cells and in BCL-6-expressing lymphomas.

Translocation of BCL-6 can, in some cases, be the sole chromosomal abnormality detectable in a diffuse large B cell lymphoma by standard cytogenetic analysis (see Mitelman, 1994), suggesting that these translocations occur early in the transformation sequence. This pivotal role for BCL-6 in lymphomagenesis is now understandable given that BCL-6 may block terminal differentiation by repressing *blimp-1* and maintain proliferation by repressing *p27kip1*. Consequently, it is worth considering whether inhibition of BCL-6 function might be a useful therapeutic strategy in some B cell non-Hodgkin's lymphomas.

Experimental Procedures

More detailed methods can be found at: http://lymphochip.nih.gov/shafferetal/.

Cell Culture, Retroviral Constructs, and Retrovirus Infection

Cell lines were maintained in RPMI 1640 media (no phenol red, GIBCO–BRL) with 10% fetal calf serum (charcoal/dextran absorbed, HyClone) and pen/strep (GIBCO–BRL). The stable WI-L2 line (WI-L2 FLERD) expressing full-length BCL-6 fused to a portion of the estrogen receptor was maintained in 1mg/ml. The Phoenix retroviral system was used to express various proteins in target cells (http://www.stanford.edu/group/nolan/phoenix_info.html). The bicistronic pBMN-IRES-Lyt2 was modified by replacing mouse Lyt2 with a puromycin resistance gene. Each retroviral construct (containing a form of *BCL-6* plus the puromycin resistance gene or the puromycin resistance gene alone to generate a control population) was transfected into the amphitrophic Phoenix packaging line to make a viral supernatant used for spin-infection (Quong et al., 1999). Transduced cells were selected with empirically determined concentrations of puromycin (Sigma). Estrogen receptor fusions with BCL-6 (FLERD and ZnFERD) were engineered (see http://lymphochip.nih.gov/shafferetal/) and induced with β-estradiol (1 μM, Sigma).

Cell Stimulation

Cells were stimulated with phorbol myristate acetate (PMA, 10 ng/ml, Sigma) and the calcium ionophore ionomycin (1 μg/ml, Sigma). Cells were also treated with goat anti-human IgM (Fab'2, Jackson Immunoresearch) and interferon-γ (10 ng/ml, R&D systems). Some cells were also treated with cycloheximide (1 μg/ml, Sigma) to arrest protein synthesis, then treated with estradiol to induce ERD-fusion proteins.

RNA Preparation and cDNA Microarray Analysis

Total RNA was prepared by the Trizol method (GIBCO–BRL). Lymphochip cDNA microarrays were prepared and hybridized as described (Alizadeh et al., 2000). Microarrays were analyzed on a GenePix (AXON) scanner at 635 nm (CY-5, red) and 523 nm (CY-3, green) wavelengths. Spot data were analyzed using software developed at the National Insitutes of Health and the programs Cluster and Treeview (M. Eisen; http://www.microarrays.org/software [Eisen et al., 1998]). Purification, stimulation, and analysis of primary human B cell populations are described in Alizadeh et al., 2000).

Details for the samples compared in Figure 2A are at http://lymphochip.nih.gov/shafferetal/.

Northern Blots and Semiquantitative RT-PCR

Northern blots were performed as described (Seyfert et al., 1996). Probes were generated from the coding regions of the human *GAPDH*, *cyclin D2*, *CD69*, *MIP-1α*, and *blimp-1* genes. cDNA for RT-PCR was generated using the cDNA Choice System (GIBCO–BRL). PCR reactions (for primers see http://lymphochip.nih.gov/shafferetal/) were performed to 25 cycles to keep amplification in the linear range.

Promoter Analysis

Portions of the human *cyclin D2* and *MIP-1α* promoters were PCR amplified, sequence confirmed, and cloned into the promoterless pGL3 luciferase reporter vector (Promega). Transient transfections were performed as described (Dent et al., 1997) using the promoter-reporters along with an excess of expression construct for BCL-6 (30 μg) or control vector (30 μg) and a β-*gal*-expressing plasmid (2 μg). Cells were harvested, and luciferase levels were measured using a luminometer. Values were normalized using β-gal production as the standard.

Flow Cytometry

Analyses were performed as described (Dent et al., 1997). With the exception of the anti-9–27/LEU13 antibody (Dr. Sharon Evans), antibodies were purchased from PharMingen. Species- and isotype-matched irrelevant antibodies were used as controls. Splenic single-cell suspensions were made by manual disruption, and mononuclear cells were separated on a Ficoll density gradient.

Immunofluoresence

For detection of FLAG-tagged BCL-6 proteins (FLERD and ZnFERD), cells were treated with EtOH (noninducer) or estradiol (inducer) for 1 hr, cytospun onto slides, and fixed for immunofluoresence. Staining was performed with isotype control or anti-FLAG antibodies, followed by a FITC-conjugated secondary antibody (Zymed).

ELISA Quantitation of Chemokine Secretion

MIP-1α levels were measured with a Quantikine Kit (R&D Systems) on a SpectraMax 250 (Molecular Devices) plate reader at 450 nm. To measure IP-10 production, we used protocols and reagents as detailed by the manufacturer (R&D Systems). Amounts of MIP-1α and IP-10 produced were calculated based on a standard curve generated from serial dilutions of recombinant human proteins (R&D Systems). Splenic B cells from heterozygous control and BCL-6 mutant littermates were prepared as above by positive selection on the basis of CD19 expression using magnetic beads (Miltenyi Biotech). B cells were placed in media with LPS (20 μg/ml, Sigma) for 3 days, supernatants were harvested, and MIP-1α levels were measured.

Cell Viability and Cell Cycle Analysis

Equal numbers of Raji control and ZnFERD-transduced cells were added to fresh media. Viability was determined by trypan blue exclusion. For cell cycle analysis, cells were harvested, resuspended in PBS, and fixed with EtOH. Cells were then placed on ice for 20 min, pelleted, and resuspended in 1 ml of 1× PBS/1% FBS. Cells were pelleted again, resuspended in a solution of 1× PBS/2.5 mM EDTA/10 μg/ml propidium iodide (Sigma) and 250 μg/ml RNase A for 40 min at 37°C, then analyzed by flow cytometry to determine the proportion of cells in each phase of the cell cycle.

Western Blotting

Western blots were performed as described (Seyfert et al., 1996). Rabbit-α-c-Myc and Rabbit-α-SP1 antibodies (Santa Cruz) were used with a secondary HRP-conjugated antibody (Amersham). Membranes were developed with ECL reagents (Amersham).

Animal Generation and Handling

Generation and handling of BCL-6 mutant mice has been described (Dent et al., 1997). Healthy mice were sacrificed between the ages of 4 and 6 weeks for analysis. Mice were maintained according to the guidelines of the National Institutes of Health Office of Animal Care and Use.

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