Brief Definitive Report

Suppression of Signal Transducer and Activator of Transcription 3–dependent B Lymphocyte Terminal Differentiation by BCL-6

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Abstract

Lymphocytes usually differentiate into effector cells within days after antigen exposure, except in germinal centers where terminal differentiation is delayed while somatic hypermutation creates high-affinity antibody mutants. Here we investigate whether arrest of terminal differentiation can be mediated by BCL-6, a transcriptional repressor that is expressed by germinal center B cells and is required for this phase of B cell development. We find that BCL-6 suppresses the differentiation of transformed and primary B cells to plasma cells by inhibiting the signal transducer and activator of transcription 3–dependent expression of the major regulator of plasma cell development, the B lymphocyte–induced maturation protein (Blimp-1). This function of BCL-6 as a repressor of B lymphocyte differentiation may also underlie the association between chromosomal translocations of its gene and B cell lymphomas.

Key words: BTB-POZ protein • plasma cell • germinal center • retrovirus • interleukins

Introduction

In T-independent B cell responses and T-dependent B cell responses not involving germinal centers, B cells rapidly differentiate into plasma cells after several days of antigen-specific clonal expansion to make antibodies available for defense against rapidly replicating pathogens. In contrast, in T-dependent B cell responses occuring in the germinal center, proliferation of antigen-specific B cells persists for weeks, suggesting that the differentiation of B cells at this site is suppressed. Suspension of terminal differentiation of B cells in the germinal center may be required to provide the conditions necessary for somatic hypermutation of V_H and V_L genes during repetitive cycles of proliferation, mutation, cell cycle arrest, and selection of B cells having higher affinity for antigens, the process that underlies the phenomenon of affinity maturation of the immune response (1, 2).

BCL-6 was initially discovered as a gene that is translocated in certain non-Hodgkin's lymphomas (3, 4). It is a sequence-specific transcriptional repressor that is expressed by germinal center B cells, but not by terminally differentiated plasma cells (5–7). DNA binding is mediated by six C2H2 Krüppel-like zinc fingers and repression by a POZ domain (8). Mice bearing homozygous disruptions of the BCL-6 locus (9–11) have a B cell–autonomous (10) absence of T-dependent germinal center formation, but preserved T-independent responses. These mice also have an inflammatory phenotype that is unrelated to the ability of BCL-6 to regulate signal transducer and activator of transcription (STAT)6-dependent transcription or to its expression by lymphocytes, but may relate to the regulation of chemokine expression in macrophages (12). A dominant negative form of BCL-6 has been shown to arrest the growth of a human Burkitt lymphoma and induce the expression of B lymphocyte–induced maturation protein 1 (Blimp-1), suggesting some role in regulating plasma cell differentiation (13). However, the dominant negative BCL-6 did not cause these cells to secrete Ig or express J chain or syndecan 1, all markers of normal plasma cells. Furthermore, although Blimp-1 is considered a regulator of plasma cell differentiation (14), it is insufficient for mediating this developmental transition in all B cell lines (15), and it has functions entirely unrelated to B cell development

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(16). Thus, a role for BCL-6 in controlling terminal B cell maturation has not been established. In this study, we demonstrate that BCL-6 suppresses cytokine-driven differentiation of a B cell line and primary B cells to plasma cells by inhibiting STAT3-dependent transcriptional events.

Materials and Methods

Retrovirus Construction and Transduction. BCL-6 cDNA was prepared from the mouse cell line A20 by reverse transcription of poly-A RNA and subcloned into the Moloney murine leukemia virus–derived vector pHL6-internal ribosome entry sequence (IRES)-green fluorescent protein (GFP) (gift of A. Venkitaraman, Cancer Reserach Campaign, Cambridge, UK) using MluI and BamHI sites. Serine-333 and -343 were mutated to alanine (17) (USE Mutagenesis kit; Amersham Pharmacia Biotech). pHL7 differs from pHL6-GFP by having a neomycin-resistance gene in place of the GFP gene. pHL6-Blimp-1-GFP was constructed by subcloning the XhoI–BglII Blimp-1 fragment from the vector pBJ1-Neo (gift of K. Calame, Columbia University, New York, NY) into SalI–BamHI-digested pHL6-GFP. pHL6–dominant negative STAT (DNSTAT)3-GFP was constructed by subcloning a DNSTAT3 (18; gift of S. Akira, Osaka University, Osaka, Japan) into the BamHI and MluI sites of pHL6-GFP. The Phoenix packaging cell line (19) was transfected with 10–15 μg of cesium-banded DNA. Supernatants were collected 48–72 h later and filtered. Lymphocytes were plated at 3×10^5 cells/well of 6-well plates. Retroviral supernatants diluted 1:1 with medium and containing 4 μg/ml polybrene were added to the cells, and the plates were centrifuged for 2 h at 1,000 *g*. After an additional 6–12 h at 37°C, the cells were washed and fresh medium was added.

Differentiation of Lymphocytes. BCL1 cells, 2.5×10^5/ml of RPMI 1640 with 15% FCS, 10 mM Hepes, and 50 μM β-mercaptoethanol, were induced to differentiate by the addition of 20 ng/ml of IL-2 and 5 ng/ml of IL-5 (R&D Systems). Medium and cytokines were replaced every 3 d, and viable cells excluding Trypan blue were counted every 2 d. Murine splenocytes were treated with anti-Thy1.2 and rabbit complement to yield 95% $CD19^+$ B cells. These were cultured for 48 h at 10^6/ml in the presence of 8 μg/ml of anti-CD40 (BD PharMingen), 20 ng/ml of IL-2, 5 ng/ml of IL-4, 5 ng/ml of IL-7, and 5 ng/ml of IL-15 (R&D Systems). After retroviral transduction, the B cells were cultured in the presence of an equivalent number of mitomycin C–treated J558L cells expressing CD40 ligand (gift of P. Lane, University of Birmingham, Birmingham, UK) and the cytokines, and stained with phycoerythrin anti–syndecan 1 (BD PharMingen). Alternatively, after retroviral transduction, 10^5 GFP^+ cells were flow sorted (Cytomation) and then returned to culture with 2×10^5 J558L cells and cytokines. For Western blot analyis, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (20), and soluble proteins were separated by electrophoresis on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, and revealed with the rabbit antibody to BCL-6 (Santa Cruz Biotechnology, Inc.) or Blimp-1 (gift of M. Davis, Stanford University, Stanford, CA) and peroxidase-conjugated murine anti–rabbit IgG. For cell cycle analysis and detection of apoptosis, BCL1 cells were fixed in 70% ethanol, treated with RNase A (0.1 mg/ml), and stained with propidium iodide (40 μg/ml). Fluorescence was measured with a FACSort™ (Becton Dickinson). Annexin V staining was carried out according to the manufacturer's instructions (Oncogene Research Products).

J chain mRNA was detected by reverse transcriptase (RT)-PCR (14). Blimp-1 mRNA was quantitated by real time quantitative RT-PCR relative to endogenous hypoxanthine phosphoribosyltransferase (HPRT) mRNA (21). The oligonucleotides used for this assay were: HPRT forward, 5′-TTAAGCAGTACAGCCCCAAAATG; HPRT reverse, 5′-CAAACTTGTCTGGAATTTCAAATCC; HPRT-Probe, 5′-FAM-CCTTTTCACCAGCAAGCTTGAAACCTTA-TAMRA; Blimp-1 forward, 5′-GGAGGATCTGACCCGAATCA; Blimp-1 reverse, 5′-CTCCACCATGGAGGTCACATC; and Blimp-1 Probe, 5′-FAM-AGCGACAATGCAGACCGTCTTGAGGACTAMRA.

IgM, IgG1, and IgA in culture supernatants were detected by ELISA using rat anti–mouse isotype-specific antibodies (BD PharMingen) as the capture antibodies and either rabbit anti–mouse IgM-horseradish peroxidase (Sigma-Aldrich) or biotinylated rat isotype-specific antibodies (BD PharMingen), followed by streptavidin-horseradish peroxidase (Pierce Chemical Co.) as

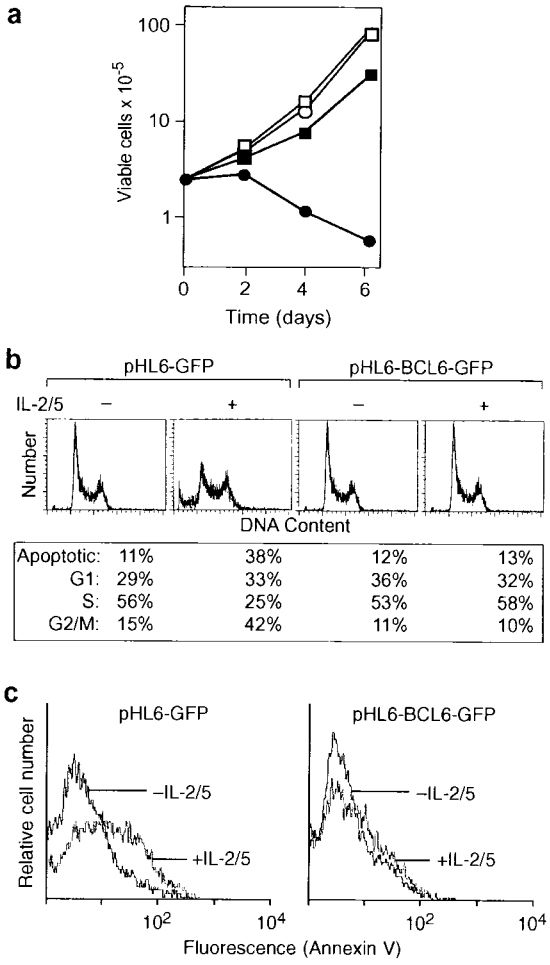

Figure 1. The effect of BCL-6 on the growth and viability of BCL1 cells. (a) The number of viable BCL1 cells transduced with pHL6-GFP (○, ●) or pHL6-BCL-6-GFP (□, ■) was determined during culture in medium lacking (○, □) or containing IL-2 and IL-5 (●, ■). (b) Cell cycle analysis of BCL1 cells cultured for 4 d in the absence or presence of cytokines was performed by flow cytometry after staining with propidium iodide. (c) Binding of annexin V to BCL1 cells cultured for 3 d in the absence or presence of cytokines was measured by flow cytometry.

the revealing antibodies. Purified antibodies (BD PharMingen) were used to construct standard curves.

STAT Assays. The STAT5 site from the IL-2 receptor α promoter (22), which contains two STAT recognition sequences, a dimerized STAT3 site from the α2 macroglobulin promoter (23) and a dimerized STAT6 site from the CD23 promoter (9), were cloned upstream of pGL3-Enhancer (Promega) into which had been previously cloned a 118-bp BamHI–NcoI fragment from the human β-globin promoter. 2×10^7 BCL1 cells were transfected by electroporation at 250 V, 960 μF (Genepulser; Bio-Rad Laboratories) with 15 μg of the luciferase reporter constructs and 5 μg of a β-galactosidase expression vector (Invitrogen). Each sample was divided into two, resuspended in medium, and cultured for 3 h. Fresh medium, either with or without 20 ng/ml of IL-2 and 5 ng/ml of IL-5, was added and culture was resumed for 24 h after which cell extracts were prepared and analyzed for protein (BCA protein assay; Pierce Chemical Co.), luciferase (Promega), and β-galactosidase (Promega). For electrophoretic mobility shift assays (EMSAs), 293T cells were transfected with an expression vector bearing the full-length wild-type mouse BCL-6 sequence, cultured for 24 h after which nuclear extracts were prepared (24). DNA probes were end-labeled with T4 polynucleotide kinase. The probes used were BCL-6 consensus (25), 5′-GAAAATTCCTAGAAAGCATA; STAT3 (α2 macroglobulin), 5′-CATCCTTCTGGGAATTCA-CAT; STAT5 (IL-2 receptor α), 5′-GTTTCTTCTAGGAAG-TAC; and STAT6 (CD23 promoter), 5′-GGTGAATTTCTAA-GAAAGG. EMSAs, cold competitor assays, and antibody-mediated inhibition were carried out as described (26).

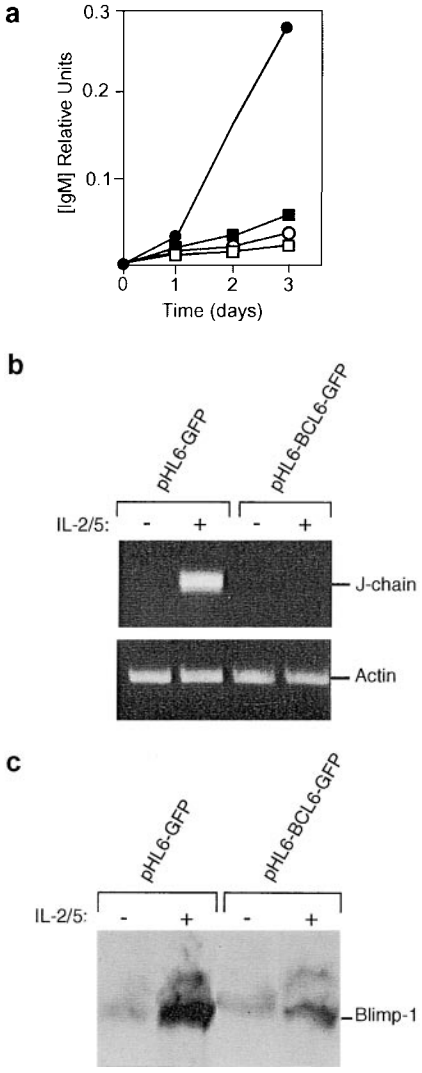

Figure 2. The effect of BCL-6 on cytokine-induced differentiation of BCL1 cells. (a) The secretion of IgM by BCL1 cells transduced with pHL6-GFP (○, ●) or pHL6-BCL-6-GFP (□, ■) during culture in medium lacking (○, □) or containing IL-2 and IL-5 (●, ■) was measured by ELISA. (b) The expression of J chain mRNA in transduced cells cultured for 3 d in the absence or presence of cytokines was assessed by RT-PCR. (c) The expression of Blimp-1 in transduced BCL1 cells cultured for 3 d in the absence or presence of cytokines was assessed by Western blot analysis.

Results and Discussion

Inhibition by BCL-6 of Plasma Cell Differentiation of BCL1 Cells. To determine whether BCL-6 can repress terminal B cell differentiation, we studied the BCL1 CW13.20-3B3 B cell line that undergoes plasma cell differentiation in response to IL-2 (27) and IL-5 (28). These cytokines cause the expression of Blimp-1 (14), a regulator of terminal B cell development that inhibits the transcription of c-myc (29) to terminate cellular proliferation and induces the synthesis of J chain and the secretion of IgM. Two BCL1 cell lines were created by the introduction of retroviral vectors that directed the expression of BCL-6 and GFP, or GFP alone, followed by flow sorting. BCL-6 was detected by Western blot analysis of lysates from cells transduced with pHL6-BCL-6-GFP at a level slightly less than that of the centroblast-like Ramos Burkitt lymphoma, but not in wild-type BCL1 cells (data not shown).

IL-2 and IL-5 altered the growth of the control BCL1 line, causing cell cycle arrest at the G2/M phase and apoptosis (Fig. 1, a–c); these effects of the cytokines were inhibited by BCL-6. IL-2 and IL-5 also caused the differentiation of the control BCL1 line to a plasma cell phenotype, as assessed by secretion of IgM, expression of J chain mRNA,

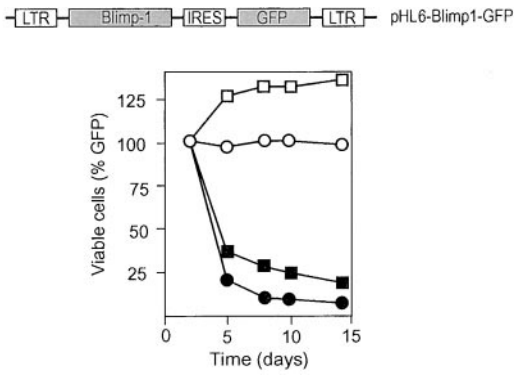

Figure 3. The effect of Blimp-1 on the growth and viability of BCL1 cells. BCL1 cells lacking (○, ●) or expressing BCL-6 (□, ■) were transduced with pHL6-GFP (○, □) or pHL6-Blimp-1-GFP (●, ■). The fraction of viable cells that were GFP^+, relative to day 2 after transduction, was determined.

and expression of Blimp-1 protein (Fig. 2, a–c). All three responses were suppressed by BCL-6.

We next determined whether the ectopic expression of Blimp-1 could overcome the capacity of BCL-6 to maintain the growth of BCL1 cells by constructing two additional retroviral vectors, pHL6-BCL-6-NEO and pHL6-Blimp-1-GFP (Fig. 3). BCL1 cells were transduced with pHL6-BCL-6-NEO and cultured in the presence of IL-2 and IL-5 to eliminate cells not expressing BCL-6. The BCL-6$^+$ cells, whose expression was confirmed by Western blot analyis (data not shown), and wild-type BCL1 cells were then transduced with pHL6-GFP or pHL6-Blimp-1-GFP, and the proportion of GFP$^+$ cells was assessed for >2 wk. The growth of BCL1 cells expressing only GFP was the same as nontransduced cells (Fig. 3). However, the expression of Blimp-1 caused the GFP$^+$ fraction to fall, regardless of the presence of BCL-6, suggesting that BCL-6 acts upstream of Blimp-1, possibly directly or indirectly suppressing the induction of Blimp-1 by cytokines.

Inhibition by BCL-6 of Plasma Cell Differentiation of Primary B Cells. To examine the effect of BCL-6 on the terminal differentiation of primary B cells, replicate samples of in vitro–activated murine splenic B cells were transduced with pHL6-GFP and pHL6-BCL-6-GFP, respectively. The B cells were then cultured in the presence of CD40 ligand and IL-2, -4, -7, and -15. CD40 ligand is required for germinal center reactions and causes B cells to become responsive to cytokines. These particular cytokines were chosen because, within the germinal center, IL-2 and IL-4 may be secreted by activated helper T cells at this site, and IL-7 and IL-15 may be produced by stromal elements, such as follicular dendritic cells. Analysis of GFP$^+$ cells on days 3 and 5 after transduction for the plasma cell marker, syndecan 1, revealed lower expression among cells having BCL-6 than among the control cells (Fig. 4 a). In addition, in these cultures containing both transduced and nontransduced B cells, the proportion that were GFP$^+$ increased among cells expressing BCL-6, but remained the same in the control cultures, indicating that BCL-6 does not inhibit the growth-promoting effects of CD40 ligand and cytokines (data not shown).

In separate experiments, transduced GFP$^+$ primary B cells were flow sorted to ~80% purity, cultured for 5 d in the presence of CD40 ligand and the same cytokines, and assayed for differentiation by Ig secretion and Blimp-1 expression. The increase in Blimp-1 mRNA and in the secretion of IgM, IgG1, and IgA in B cells expressing BCL-6 was reduced to 20–40% of the levels seen with the GFP only, control cells (Fig. 4, b and c). The predominant isotype, regardless of the presence of BCL-6, was IgG1, presumably reflecting isotype switching induced by IL-4. Therefore, BCL-6 can suppress terminal differentiation of primary B cells.

BCL-6, STAT3, and Plasma Cell Differentiation. IL-2 and IL-5 activate STAT3 and STAT5a/b (for a review, see reference 30), and the BCL-6 consensus DNA-binding site is similar to that for STATs (9). However, BCL-6 has been shown only to bind to STAT6 sites from the CD23 promoter (9) and the germline Iε promoter (25). To determine whether BCL-6 might also regulate STAT3- or STAT5-dependent transcription, luciferase reporter constructs containing a dimerized STAT3 site from the α2-macroglobulin promoter or a dimerized STAT5 site from the IL-2 receptor α chain promoter, were transiently expressed in the control and BCL-6$^+$ BCL1 cell lines; the cells were activated by IL-2 and IL-5. BCL-6–inhibited cytokine induced transcription from the STAT3 reporter, but not the STAT5 reporter (Fig. 5 a). The interaction of BCL-6 with STAT sites was further evaluated by gel mobility shift assays in which BCL-6 was shown to form protein–DNA

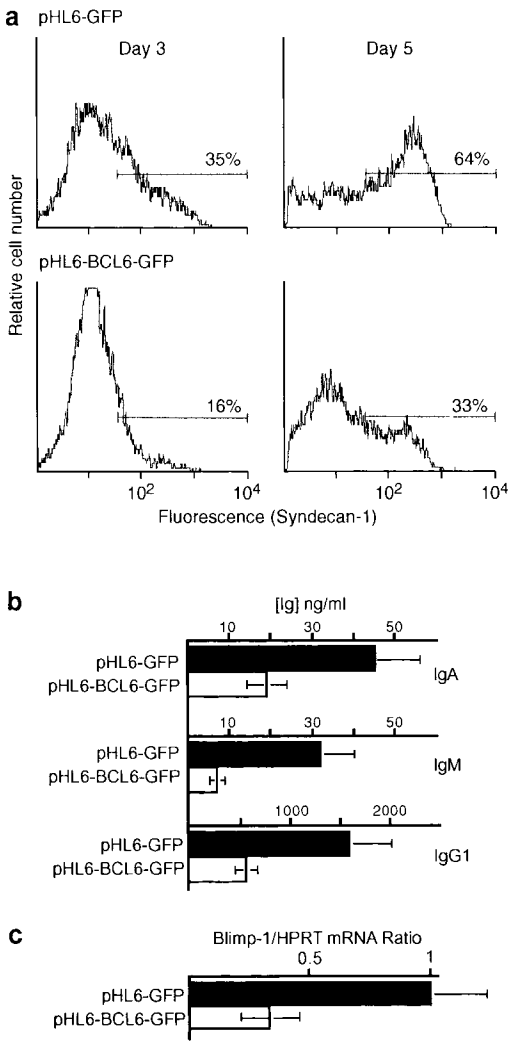

Figure 4. The effect of BCL-6 on the differentiation of primary B cells. (a) Primary B cells transduced with pHL6-GFP or pHL6-BCL-6-GFP were cultured in the presence of CD40 ligand and IL-2, -4, -7, and -15, and GFP$^+$ cells were assessed for expression of syndecan 1 by flow cytometry. (b) The secretion of IgM, IgG1, and IgA by flow-sorted primary B cells transduced with pHL6-GFP or pHL6-BCL-6-GFP after culture for 5 d in the presence of CD40 ligand and IL-2, -4, -7, and -15 was measured by ELISA. (c) The expression of Blimp-1 mRNA relative to HPRT mRNA by flow-sorted primary B cells, which had been transduced with pHL6-GFP or pHL6-BCL-6-GFP and cultured with CD40 ligand and IL-2, -4, -7, and -15, was determined on day 5.

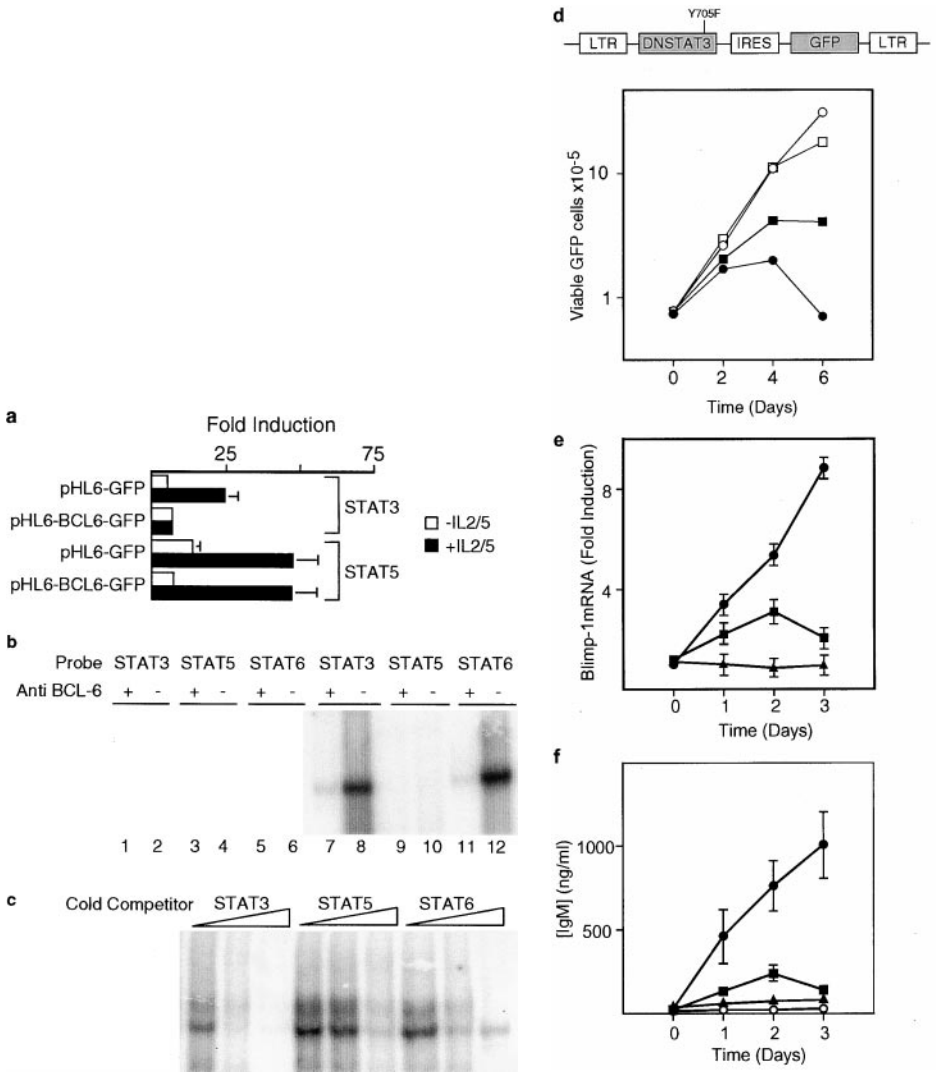

Figure 5. STAT3 and regulation by BCL-6 of the differentiation of BCL1 cells. (a) Transduced BCL1 cells were transfected with luciferase reporter constructs having STAT3 or STAT5 sites, cultured in the absence or presence of cytokines, and assessed for luciferase activity relative to the activity of cells transfected with a construct lacking a STAT site. (b) Nuclear lysates from untransfected (lanes 1–6) and BCL-6–transfected 293T fibroblasts (lanes 7–12) were incubated with control antiserum or anti–BCL-6 followed by addition of ^{32}P-oligonucleotide probes having STAT sites, and subjected to gel mobility shift assay. (c) Nuclear lysates having BCL-6 were incubated with a ^{32}P-oligonucleotide probe having a consensus BCL-6 binding site in the absence or presence of 1, 5, or 50 pmol of unlabeled, competitor probes having the designated STAT sites, and subjected to gel mobility shift assay. (d) The number of viable BCL1 cells transduced with pHL6-GFP (○, ●) or pHL6-DNSTAT3-GFP (□, ■) was measured during culture in medium lacking (○, □) or containing IL-2 and IL-5 (●, ■). (e) The expression of Blimp-1 mRNA compared with HPRT mRNA, in BCL1 cells transduced with pHL6-GFP (●), pHL6-BCL-6-GFP (▲), or pHL6-DNSTAT3-GFP (■) was measured by real time quantitative RT-PCR during culture in medium containing IL-2 and IL-5, and is expressed relative to the level in pHL6-GFP BCL1 cells just before the addition of cytokines. (f) The secretion of IgM by BCL1 cells transduced with pHL6-GFP (○, ●), pHL6-BCL-6-GFP (▲), or pHL6-DNSTAT3-GFP (■) during culture in medium lacking (○) or containing IL-2 and IL-5 (●, ▲, ■) was measured by ELISA.

complexes with radiolabeled STAT3 and STAT6 binding site probes that were inhibited by antibody to BCL-6. BCL-6 did not form a complex with the STAT5 probe (Fig. 5 b). Furthermore, unlabeled oligonucleotides having STAT3 or STAT6 binding sites competed with a labeled probe having the consensus sequence recognised by BCL-6, whereas the oligonucleotide having a STAT5 binding site was relatively less effective (Fig. 5 c). Since IL-2 alone, but not IL-5, can induce differentiation of BCL1 cells, and IL-2, but not IL-5, activates STAT3, the finding that BCL-6 represses STAT3-dependent transcription was consistent with the means by which terminal differentiation of B cells is suppressed by BCL-6.

To provide direct evidence for an essential role of STAT3 in BCL1 differentiation, a retroviral vector was constructed, pHL6-DNSTAT3-GFP, directing the expression of a dominant negative form of this transcription factor (18). BCL1 cells were transduced with this or a control vector, pHL6-GFP, and the number of viable GFP$^+$ cells was measured during culture in the absence or presence of IL-2 and IL-5. The cytokine-induced decrease in viability observed with control cells was attenuated in cells expressing DNSTAT3 (Fig. 5 d). The control and DNSTAT3 BCL1 cells were isolated by cell sorting and compared with BCL-6–expressing BCL1 cells during culture in the presence of IL-2 and IL-5 for the induction of Blimp-1 and secretion of IgM. The increase in Blimp-1 mRNA, as measured by real time quantitative RT-PCR, and IgM secretion was suppressed by DNSTAT3, although not as effectively as by BCL-6 (Fig. 5, e and f). Thus, STAT3 is essential for cytokine-induced terminal differentiation of B cells, suggesting that the ability of BCL-6 to regulate STAT3-dependent transcription is responsible for its suppression of differentiation.

This study shows that a potential physiological function for BCL-6 is the inhibition of differentiation of B cells to plasma cells in the germinal center, allowing affinity maturation of the immune response to occur. This function may account for the inability of mice lacking BCL-6 to form germinal centers, since in its absence, activated B cells

would terminally differentiate rather than accumulate as centrocytes and centroblasts. This effect of BCL-6 is caused, at least in part, by the repression of STAT3 elements involved in Blimp-1 expression. One might anticipate that there may be other STAT3 sites which mediate the growth-promoting function of activated STAT3 and are not affected by BCL-6, thereby enabling this transcriptional repressor to bias the cytokine response of germinal center B cells towards growth rather than terminal differentiation. Finally, this study offers an explanation for the oncogenic potential of BCL-6 since its constitutive expression through chromosomal translocations would block terminal differentiation and maintain a proliferating, mutating germinal center B cell pool, thereby increasing the likelihood of other genetic events associated with the development of B cell lymphomas.

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