Repression of *c-myc* Is Necessary but Not Sufficient for Terminal Differentiation of B Lymphocytes In Vitro

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The importance of *c-myc* as a target of the Blimp-1 repressor has been studied in BCL-1 cells, in which Blimp-1 is sufficient to trigger terminal B-cell differentiation. Our data show that Blimp-1-dependent repression of *c-myc* is required for BCL-1 differentiation, since constitutive expression of c-Myc blocked differentiation. Furthermore, ectopic expression of cyclin E mimicked the effects of c-Myc on both proliferation and differentiation, indicating that the ability of c-Myc to drive proliferation is responsible for blocking BCL-1 differentiation. However, inhibition of c-Myc by a dominant negative form was not sufficient to drive BCL-1 differentiation. Thus, during Blimp-1-dependent plasma cell differentiation, repression of *c-myc* is necessary but not sufficient, demonstrating the existence of additional Blimp-1 target genes.

In many cell lineages, the c-Myc oncoprotein is critical for regulating growth control, apoptosis, and/or differentiation, and its dysregulation also plays a causal role in a wide variety of neoplasias (12, 17, 52). c-Myc is induced by mitogens expressed during G₁ to S progression and required for efficient cell cycle progression (reviewed in references 12 and 17). Ectopic expression of c-Myc promotes reentry of fibroblasts into the cell cycle in the absence of mitogenic stimulation (18). c-Myc antisense oligonucleotides inhibit entry into S phase (24), and antagonism of c-Myc by Mad overexpression inhibits proliferation (54). Recent studies suggest that c-Myc's proliferative effects may be due primarily to activation of genes required for cell growth rather than genes that regulate cell cycle progression (28, 29), although both kinds of targets probably play a role in c-Myc-dependent proliferation. c-Myc expression also induces apoptosis when mitogen is withdrawn from fibroblasts and in other cell contexts (20, 49). Downregulation of c-Myc accompanies differentiation (19, 34), and ectopic expression of c-Myc blocks terminal differentiation in mouse erythroleukemia cells, human monoblastic cells (U-937), myeloid leukemic M1 cells, and postmitotic murine keratinocytes (13, 25, 26, 35, 50). Additionally, inhibition of c-Myc results in induction of differentiation in a human promyelocytic leukemia cell line (HL-60) and in murine F9 teratocarcinoma cells (23, 25, 73) or reverse tumorigenesis in hematopoietic lineages of mice (21).

c-Myc belongs to the basic helix-loop-helix/leucine zipper family of transcription factors (7). Heterodimerization between c-Myc and its partner, Max, is obligatory for binding site-dependent activation of target genes (6, 7, 53). Genes containing Myc-Max binding sites have been identified and shown to be regulated by Myc-Max (14). In addition, c-Myc regulates transcription of other genes that lack Myc-Max binding sites, by binding at initiator sequences (38) or by associating with other transcription proteins, including TFII-I (60), YY-1 (64–66), and Miz-1 (62).

Consistent with its role in other cell lineages, c-Myc has been shown to be important in normal B-cell lymphopoiesis and to

be dysregulated in many B-cell malignancies. During B-cell development, c-Myc levels change in precise ways, suggesting that c-Myc is critical for the highly regulated periods of cell proliferation that occur as B cells mature (45). c-Myc is present at high levels in pro-B, pre-BI, and pre-BII cells and falls when B cells become small, resting, and surface immunoglobulin M (IgM)-positive immature B cells. c-Myc levels rise again during antigen-induced proliferation of mature B cells (45). Dysregulated expression of c-Myc in B cells is often tumorigenic. For example, chromosomal translocations of the *c-myc* gene to Ig gene loci are present in most human Burkitt's lymphomas and murine plasmacytomas (37), and transgenic mice expressing *c-myc* under the control of the E μ heavy-chain intronic enhancer develop progressive, stage-nonrestricted B-lymphoid neoplasias (1) preceded by increased cell size of pretransformed B cells (28). Finally, c-Myc expression declines as B cells differentiate into memory cells (42) or Ig-secreting plasma cells (36). Although the importance of c-Myc for growth control during normal B lymphopoiesis and in B-cell tumors is clear, the significance, if any, of *c-myc* repression during terminal B-cell differentiation has not been carefully investigated.

The zinc finger protein Blimp-1 (B lymphocyte-induced maturation protein-1) represses *c-myc* in B cells. Blimp-1 was first identified as a plasmacytoma-specific repressor factor that bound to a negative element in the murine *c-myc* promoter (30, 39). Plasmacytoma-specific repressor factor was subsequently identified as Blimp-1 (39). Blimp-1 was cloned by subtractive hybridization of mRNAs induced when a mature B-cell lymphoma line, BCL-1, differentiated into a plasma cell phenotype in response to treatment with interleukin-2 (IL-2) and IL-5 (71). Blimp-1 is also induced in other in vitro models of terminal B-cell differentiation and is expressed in murine plasmacytoma lines, human myeloma lines, and in vivo human and murine plasma cells (3a, 39, 55, 69; J. F. Piskurich et al., submitted for publication).

Significantly, ectopic expression of Blimp-1 is sufficient to drive differentiation of BCL-1 cells to a plasma cell phenotype, evidenced by induction of Syndecan-1 on the cell surface, J-chain expression, IgM secretion, and increased cell size and granularity (39, 71). Ectopic expression of Blimp-1 also downregulates endogenous c-Myc, correlating well with the cessation of proliferation that occurs during B-cell terminal differentiation (39). Inhibition of B-cell differentiation by CD40

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engagement is associated with downregulation of Blimp-1 induced by cytokines in CH12 cells (55), further supporting the role of Blimp-1 in terminal differentiation of B cells. Thus, Blimp-1 appears to be a master regulator of terminal B-cell differentiation.

Given the established importance of c-Myc in growth regulation and differentiation in other cell lineages, it seemed likely that the previously observed Blimp-1-dependent repression of *c-myc* transcription was critical for terminal B-cell differentiation. We wished to determine if c-myc repression was necessary and/or sufficient for plasma cell development, and if either were true, we wished to determine which aspect of c-Myc function was important in controlling plasma cell differentiation.

To address these questions experimentally, we have taken advantage of the BCL-1 culture model for plasma cell differentiation. The ability of BCL-1 lymphoma cells to differentiate to a plasma cell phenotype was used early on to study expression of secreted versus membrane forms of *mu* mRNA in response to lipopolysaccharide (LPS) (75) and to identify and characterize factors for inducing plasma cell differentiation (8). Subsequently, BCL-1 cells have been exploited to study other aspects of plasma cell development, including induction of *J chain* transcription (44) and the identification of BSAP as an IL-2-inducible repressor of *J chain* transcription (59). Blimp-1 was originally cloned and characterized based on its ability to trigger differentiation of BCL-1 cells (46, 71). Therefore, BCL-1 cells provide a well-characterized model system for further analysis of the role of Blimp-1 in plasma cell differentiation.

We report here that ectopic expression of c-Myc blocks terminal differentiation of BCL-1 cells, showing that repression of *c-myc* by Blimp-1 is necessary for differentiation of mature B cells into plasma cells. Furthermore, the importance of repressing c-Myc is related to the ability of c-Myc to promote proliferation, since ectopic expression of cyclin E, which mimics the proliferative effect of c-Myc but not other effects, also inhibits terminal differentiation of BCL-1 cells. However, inhibition of c-Myc by induction of a dominant negative form of c-Myc was not sufficient to cause differentiation of BCL-1 cells, although it was sufficient to inhibit proliferation. These data suggest that repression of *c-myc* transcription and cell cycle arrest are not sufficient to cause BCL-1 differentiation and indicate that Blimp-1-dependent regulation of additional target genes is required for full plasma cell differentiation.

MATERIALS AND METHODS

Cell culture and BCL-1 cell differentiation. BCL-1 cells (CW13.20-3B3, ATCC CRL 1669) were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Product, Inc.), gentamicin (20 µg/ml) (Gemini), and 50 µM β-mercaptoethanol. To induce differentiation, 2 ml of cells (10⁵ cells/ml) was placed in six-well plates and either left untreated or treated with recombinant mouse IL-2 and IL-5 (20 ng/ml) (R&D systems, Inc.) for 3 days. For induction of dominant negative c-Myc, 4-hydroxytamoxifen (4-OHT) (Sigma) dissolved in ethanol was added directly to culture medium to a final concentration of 500 nM. Lovastatin (LOV) (Calbiochem), for inhibiting the cell cycle, was dissolved in H₂O.

Plasmids. The retroviral vector pGC-Blimp-YFP was cloned by blunt-ended ligation of a cDNA fragment of hemagglutinin (HA)-tagged Blimp-1 into the *Clal* site of pGC-IRES-YFP (a kind gift from G. Fathman, Stanford). pSV2Myc and its control vector pSV2neo were described previously (39). For constructing the cyclin E expression vector, mouse cyclin E cDNA was cut with *EcoRI* from pSKmycE and then ligated into the *EcoRI* site of pcDNA3.1Zeo (Invitrogen). For constructing the inducible dominant negative c-Myc expression vector, the cDNA of dominant negative human c-Myc fused to the mouse estrogen receptor (MycDN-ER) was cut with *EcoRI* from pBabeMycDNERpuro (a gift from G. Littlewood [40]) and ligated into the *EcoRI* site of pcDNA3.1Zeo.

Transfection and transduction. Various retroviral vectors (10 µg) were transfected into Phoenix cells (a kind gift from G. Nolan, Stanford) using the calcium phosphate method. Virus supernatant was collected 48 h posttransfection and

prepared essentially as previously described (10). Virus-infected cells were sorted by fluorescence-activated cell sorting (FACS) for yellow fluorescent protein (YFP) expression.

RT-PCR. Reverse transcription (RT)-PCR on virus-transduced cells was performed essentially as previously described (4). Briefly, 250 ng of total RNA, isolated by the Trizol method (Gibco-BRL) from YFP⁺ cells, was digested with 10 U of DNase (Promega) for 1 h and subjected to cDNA synthesis. Mouse c-Myc was amplified using primers 5'-GGGCCAGCCCTGAGCCCTAGTG C-3' and 5'-ATGGAGATGAGCCCGACTCCGACC-3'. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using primers 5'-TTAGCACCCTGGCCAAGG-3' and 5'-CTTACTCCTTGGAGGCCATG-3'. Cycling conditions were 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C for 30 cycles. PCR products were analyzed on a 1.5% agarose gel.

BCL-1 transfectants stable transfectants. A total of 10⁷ BCL-1 cells in log phase were transfected with 10 µg of experimental expression vectors or control vector by electroporation as previously described (39). Forty-eight hours after transfection, cells were placed in 96-well plates at limiting dilution and treated with geneticin (800 µg/ml) (Gibco-BRL) for selection of c-Myc and control transfectants or zeocin (400 µg/ml) (Invitrogen) for selection of cyclin E stable transfectants, inducible dominant negative c-Myc stable transfectants, and control transfectants. Cells were fed weekly with fresh medium containing antibiotics, and resistant clones were expanded for analysis.

Western blot analysis. Whole-cell lysates were prepared in 20 mM Tris-HCl (pH 7.5)–10% glycerol–150 mM NaCl–1% NP-40–0.1% sodium dodecyl sulfate (SDS)–0.5% deoxycholate–2 mM dithiothreitol (DTT)–1 mM phenylmethylsulfonyl fluoride (PMSF)–protease inhibitor cocktail (Sigma) and centrifuged in a cold room at 13,000 rpm for 5 min. Supernatants were aliquoted and frozen for protein quantification and subsequent SDS-polyacrylamide gel electrophoresis (PAGE), and 20 µg of protein, quantified by the Bradford assay (Bio-Rad), was separated on an SDS–8% PAGE gel (for monitoring the expression of c-Myc and cyclin E) or SDS–6% PAGE gel (for monitoring the expression of MycDNER) and proteins were then electrotransferred onto a nitrocellulose membrane. Membranes were blocked with 5% dry milk in phosphate-buffered saline (PBS) plus 0.2% Tween 20 (PBS-T). Subsequently, membranes were blotted with a polyclonal anti-mouse c-Myc (39) diluted at the ratio of 1:3,000, polyclonal anti-cyclin E (Santa Cruz Biotechnology; M-20) diluted 1:100, monoclonal anti-human c-Myc (Santa Cruz Biotechnology; 9E10) diluted 1:100, or monoclonal anti-mouse β-actin (Sigma) diluted 1:3,000 in 2% dry milk–PBS-T. A peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim) or goat anti-mouse IgG (Boehringer Mannheim) was later used as the secondary antibody at a dilution of 1:10,000 in 2% dry milk–PBS-T. Subsequent enhanced chemiluminescence (NEN Life Science Product) was performed according to the manufacturer's suggestions.

RNAse protection assay. Total cellular RNA was isolated by the guanidinium thiocyanate procedure (11). RNase protection assays were performed as described (41). Briefly, antisense cRNA probes were generated using T3 or T7 RNA polymerase (Promega) with [α-³²P]UTP from cDNA templates, and 10 µg of total RNA was then hybridized with a 360-bp probe specific for the zinc finger 1 and 2 regions of mouse Blimp-1 and a 180-bp mouse GAPDH probe in 80% formamide–40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] buffer (pH 6.4)–400 mM NaCl–1 mM EDTA overnight at 45°C. The reaction was then treated with RNase cocktail (Amicon) at 30°C for 30 min followed by treatment with 0.125 mg of proteinase K and 0.5% SDS at 37°C for 15 min. The RNA was analyzed on a denaturing 6% polyacrylamide–urea gel. Gel imaging was done by PhosphorImager, and band intensity was analyzed by ImageQuant software (Molecular Dynamics).

Flow cytometry. For surface Syndecan-1 analysis, cells were harvested, washed once with PBS, and suspended in PBS containing 2% fetal bovine serum at 10⁶ cells/ml. Cells were then incubated with phycoerythrin-conjugated anti-mouse Syndecan-1 (PharMingen) at 4°C for 30 min (1 µl of antibody per 10⁵ cells), washed two times with PBS, and then analyzed by FACSscan (Becton Dickinson) and CellQuest software.

Cell cycle analysis. 5-Bromo-2'-deoxyuridine (BrdU) (Boehringer Mannheim) was added to cells at a final concentration of 10 µM for 2 h, and cells were harvested, washed once with PBS, and fixed in 70% ethanol for at least 12 h. Staining was performed according to the manufacturer's instructions (PharMingen). In brief, 10⁶ cells/ml were denatured in 2 N HCl, neutralized by 0.1 M sodium borate (pH 8.5), and then stained with fluorescein isothiocyanate-conjugated mouse anti-human BrdU antibody (PharMingen) at room temperature for 30 min. After staining with propidium iodide (10 µg/ml) (Boehringer Mannheim) in PBS for 20 min at room temperature, cells were analyzed using FACSscan.

ELISA. For the enzyme-linked immunosorbent assay (ELISA), cell supernatants were harvested by centrifugation of the culture medium from treated or untreated BCL-1 cells twice at 2,000 rpm. Supernatants were then serially diluted in duplicate in PBS containing 1% bovine serum albumin into 96-well plates coated with anti-mouse IgM (Southern Biotechnology Associates) and incubated for 1.5 h at room temperature. Captured IgM was further incubated with alkaline phosphatase-conjugated goat anti-mouse IgM (Southern Biotechnology Associates) diluted to 1:1,000 in PBS containing 1% bovine serum albumin for 1 h at room temperature. The alkaline phosphatase substrate *p*-nitrophenyl phosphate (Southern Biotechnology Associates) was dissolved according to the manufac-

FIG. 1. Ectopic expression of Blimp-1 decreases endogenous *c-myc* transcription. BCL-1 cells (2×10^5) were infected with pGC-YFP or pGC-Blimp-YFP. Three days postinfection, cells were sorted, and RNA was prepared from YFP-positive cells and used for semiquantitative RT-PCR analysis. cDNA dilutions, as indicated, were analyzed for *c-myc* and *GAPDH* mRNAs.

turer's instructions at the concentration of 1 mg/ml. The plates were subsequently read on a plate reader (Molecular Devices). Mouse IgM (Southern Biotechnology Associates) was used for generating the standard curve.

RESULTS

Ectopic expression of c-Myc blocks terminal differentiation of BCL-1 cells in response to cytokine. Ectopic expression of Blimp-1 is sufficient to cause terminal differentiation to a plasma cell phenotype in the BCL-1 model system (39, 71). We have previously shown that one target of Blimp-1-dependent transcriptional repression is the *c-myc* gene (39). *c-Myc* levels fall in BCL-1 cells upon induction of Blimp-1 (39). In splenocytes treated with lipopolysaccharide, Blimp-1 mRNA is induced and *c-Myc* levels decrease (3). To extend these findings, we monitored endogenous *c-myc* mRNA levels in BCL-1 cells expressing ectopic Blimp-1 using a retrovirus expressing a bicistronic mRNA for Blimp-1 and YFP. Three days after virus infection, about 50% of the cells were infected, as determined by FACS for YFP expression. *c-myc* mRNA was determined by semiquantitative RT-PCR in YFP⁺ BCL-1 cells infected with either Blimp-1-YFP virus or YFP-only control virus. *GAPDH* levels were used as an internal control for cDNA template. The data show that ectopic expression of Blimp-1 was sufficient to cause a decrease in endogenous *c-myc* mRNA of approximately fourfold (Fig. 1), formally demonstrating that Blimp-1 represses endogenous *c-myc* transcription in BCL-1 cells.

Since Blimp-1 represses *c-myc* and induces plasma cell differentiation in BCL-1 cells, we took advantage of the BCL-1 system to assess the functional importance of repressing *c-myc* transcription in the context of plasma cell differentiation. Stable transfectants of BCL-1 cells expressing c-Myc driven by a constitutive simian virus 40 promoter (Myc-BCL-1) were generated for this purpose. Stable transfectants were then treated with IL-2 and IL-5 to induce differentiation. As shown in Fig. 2A, after 3 days of IL-2 and IL-5 treatment, c-Myc declined to 60%. In two c-Myc-expressing cell lines, little or no decrease was observed following cytokine treatment: c-Myc declined to 84% in Myc#2 and remained at 100% in Myc#5. Similar results were found by analyzing *c-myc* mRNA levels in control and c-Myc transfectants (not shown).

Following treatment with IL-2 and IL-5, BCL-1 cells undergo terminal differentiation into plasma cells, characterized by cessation of cell proliferation, induction of surface marker Syndecan-1, and IgM secretion (39, 46, 71). We examined the effect of ectopic c-Myc expression on these phenotypic hallmarks of terminal differentiation. To analyze the cell cycle status of c-Myc and control transfectants, cells were treated

FIG. 2

with IL-2 and IL-5 for 3 days, pulsed with BrdU to monitor DNA replication, and stained with propidium iodide to monitor DNA content prior to analysis by FACS. In two control transfectants, after 3 days of IL-2 and IL-5 treatment, cells became quiescent (percentage of cells in S phase dropped from 25 to 5% in control #1 and from 44 to 16% in control #2), consistent with other studies showing that B cells undergo growth arrest during terminal differentiation (22) (Fig. 2B). In contrast, Myc-BCL-1 cells continued proliferating after 3 days of cytokine treatment; the percentage of cells in S phase was essentially the same as that of untreated cells (28% versus 25% in Myc#2, and 39% versus 43% in Myc#5) (Fig. 2B).

We next assayed the ability of these cells to differentiate following cytokine treatment by measuring the levels of secreted IgM and the plasma cell surface marker Syndecan-1. We found that ectopic expression of c-Myc blocks IgM secretion in response to IL-2 and IL-5 treatment. As shown in Fig. 2C, a control transfectant (#2) showed significant IgM production, but Myc-BCL-1 cells (#1 to #5) failed to secrete IgM. Syndecan-1 on the cell surface was measured by flow cytometry; its level increased about threefold in a control transfectant after 3 days of cytokine treatment (Fig. 2D, top panel, compare fine line to dark line). In contrast, Syndecan-1 remained unchanged in Myc-BCL-1 lines (#2 and #5 are shown) (Fig. 2D, middle and bottom panels). These data show that ectopic expression of c-Myc blocks terminal differentiation of BCL-1 cells, indicating that repression of *c-myc* by Blimp-1 is a necessary event for plasma cell differentiation in this culture model.

Ectopic expression of cyclin E blocks terminal differentiation of BCL-1 cells in response to cytokine. c-Myc downregulation, accompanied by growth arrest, has been shown to be associated with terminal differentiation in many cell lineages in addition to B cells (16, 25, 34, 36, 57). However, the precise mechanism(s) by which c-Myc functions in terminal differentiation is not well understood. The BCL-1 system provides a way to explore this question because we can test which aspect of c-Myc function is responsible for the inhibition of BCL-1 terminal differentiation. One important facet of c-Myc function is to promote proliferation, either by regulating genes encoding cell cycle regulators or by regulating genes involved in growth and metabolism (14). However, c-Myc also regulates genes such as telomerase reverse transcriptase, albumin, Ig lambda, and terminal deoxynucleotidyl transferase that have no obvious role in proliferation (14). We reasoned that if the proliferation aspect of c-Myc's activity was critical for blocking BCL-1 differentiation, it should be possible to achieve the same effect by activating proliferation in a different way. Alternatively, if regulation of other c-Myc-dependent target genes was required, enforced proliferation would not be sufficient to inhibit BCL-1 terminal differentiation.

Cyclin E has been shown to promote the G₁ phase of the cell cycle (33) and to abrogate cell cycle arrest induced by overexpression of p16^{INK4a}, a cdk4 inhibitor (2). Thus, we generated stable BCL-1 cell lines constitutively expressing mouse cyclin E (cyclin E-BCL-1) driven by a cytomegalovirus promoter in

B.

% in S phase

C.

IgM (ng/ml)

D.

FIG. 2. Effects of ectopic expression of c-Myc on BCL-1 terminal differentiation. (A) Total c-Myc levels did not decline in Myc-BCL-1 transfectants in response to cytokine treatment. Two Myc-BCL-1 lines (Myc#2 and Myc#5) and a control transfectant (#2) growing in log phase were either treated with recombinant IL-2 and IL-5 for 3 days or left untreated. Immunoblots to detect c-Myc and β -actin in cytokine-treated (+) and nontreated (–) cells are shown. (B) Percentage of cells in S phase analyzed by FACS analysis. Cells (two control transfectants, #1 and #2, and two Myc-BCL-1 lines, #2 and #5) with or without IL-2 and IL-5 treatment for 3 days were subjected to BrdU and propidium iodide staining. One experiment, representative of three, is shown. (C) Secreted IgM analyzed by ELISA from culture supernatants after 3 days of cytokine treatment (solid bar) or untreated (open bar). Experiments were performed at least three times, and standard deviations are shown. (D) FACS profiles of surface Syndecan-1 staining of control transfectant (#2) and Myc-BCL-1 lines (#2 and #5) treated with IL-2 and IL-5 for 3 days (dark line) or left untreated (fine line). Experiments were done at least four times, and one set of representative profiles is shown.

order to keep BCL-1 cells in a proliferative state after IL-2 and IL-5 treatment. Immunoblots showed that while endogenous cyclin E was undetectable in control cells, cyclin E was clearly detectable in three cyclin E-BCL-1 lines (#4, #7, and #9) chosen for study (Fig. 3A). Cell cycle analysis, carried out as described in the legend to Fig. 3B, showed that a control transfectant showed a significant reduction in the percentage of cells in S phase following cytokine treatment (14 versus 41% in S phase) (Fig. 3B). However, the cytokine-treated cyclin E-BCL-1 lines behaved like the cytokine-treated Myc-BCL-1 lines and continued to cycle (Fig. 3B). Similar results were obtained in two additional independent experiments. These data show that ectopic expression of cyclin E mimics ectopic expression of c-Myc in BCL-1 cells with respect to keeping the cells in cycle following cytokine treatment.

Having established that both the Myc- and cyclin E-BCL-1 lines continued to proliferate following cytokine treatment, we asked whether cyclin E expression mimicked c-Myc with respect to inhibition of terminal differentiation. Interestingly, after 3 days of IL-2 and IL-5 treatment, we observed significant IgM secretion (increased up to about 10-fold) in a control transfectant. However, IgM secretion was dramatically impaired in all cyclin E-BCL-1 lines (#4, #7, and #9) (Fig. 3C). When the plasma cell surface marker Syndecan-1 was monitored by FACS (Fig. 3D), we observed only a slight increase in the cyclin E-BCL-1 lines compared to a control transfectant, in which surface Syndecan-1 increased about threefold.

In Table 1, the phenotypic characteristics of Myc-BCL-1 and cyclin E-BCL-1 lines after 3 days of IL-2 and IL-5 treatment are summarized and compared. We conclude that ectopic expression of cyclin E mimics ectopic expression of c-Myc and inhibits BCL-1 terminal differentiation. These data strongly support the view that it is the ability of c-Myc to drive proliferation that inhibits BCL-1 differentiation, and this aspect of c-Myc function must be repressed during B-cell differentiation.

Blimp-1 mRNA is induced by cytokine treatment of Myc- and cyclin E-BCL-1 cells. We and others have shown that IL-2 plus IL-5 induces *Blimp-1* mRNA in BCL-1 cells (39, 71) and that Blimp-1 is sufficient to drive their terminal differentiation. One potential explanation for the failure of Myc- and cyclin E-BCL-1 cells to differentiate is that *Blimp-1* mRNA induction might be blocked. Therefore we examined the induction of *Blimp-1* mRNA in these cells and in parental BCL-1 cells using an RNase protection assay (Fig. 4). In parental BCL-1 cells, the induction of *Blimp-1* mRNA was detected after 2 h of IL-2 plus IL-5 treatment, and after 48 h, *Blimp-1* mRNA was induced 2.7-fold. *Blimp-1* mRNA increased similarly following cytokine treatment of the transfectants. Induction was observed after 2 h of cytokine treatment, and after 48 h Myc-BCL-1 line (#5) showed 5.3-fold induction and cyclin E-BCL-1 line (#7) 3.5-fold induction. Thus, induction of *Blimp-1* mRNA in response to cytokine treatment occurs normally in lines ectopically expressing c-Myc or cyclin E. These data show that failure to induce *Blimp-1* mRNA cannot explain the failure of the c-Myc- and cyclin E-expressing lines to differentiate. They also show that *Blimp-1* mRNA levels are not subject to negative feedback by c-Myc or by proliferation.

We also examined c-Myc in the cyclin E-BCL-1 lines using immunoblotting. As shown in Fig. 5, in a control transfectant treated with IL-2 and IL-5 for 3 days, c-Myc protein levels decreased to 46% of those in untreated cells. c-Myc levels in all of the cyclin E BCL-1 lines (#4, #7, and #9) decreased similarly after treatment with cytokine (47, 50, and 39%, respectively). This shows that ectopic expression of cyclin E does not affect the normal decrease in c-Myc which occurs in response to cytokine-dependent induction of the Blimp-1 transcriptional

FIG. 3. Ectopic expression of cyclin E promotes proliferation and blocks BCL-1 differentiation after cytokine treatment. (A) Immunoblots for cyclin E and β -actin from the cell lysates of cyclin E-BCL-1 (#4, #7, and #9) and a control transfectant. (B) Percentage of cells in S phase as analyzed by BrdU and propidium iodide staining from 2×10^5 log-phase cells (control transfectant, Myc-BCL-1, and cyclin E-BCL-1 cells) treated with cytokine for 3 days (solid bar) or left untreated (open bar). Experiments were done at least twice, and one representative result is shown. (C) Secreted IgM measured by ELISA for control and cyclin E-BCL-1 (#4, #7, and #9) cells treated with IL-2 and IL-5 for 3 days (solid bar) or left untreated (open bar). Standard deviations were derived from three independent experiments. (D) FACS profiles of Syndecan-1 staining for control transfectant and cyclin E-BCL-1 (#4, #7, and #9) with (dark line) or without (fine line) cytokine treatment for 3 days. Experiments were done at least twice.

repressor. It provides further evidence that normal regulation of c-Myc targets not related to cell cycle progression is insufficient to inhibit BCL-1 differentiation in conditions of enforced proliferation.

Expression of dominant negative c-Myc is not sufficient to drive terminal differentiation of BCL-1 cells. Our data show that repression of c-myc by Blimp-1 is necessary for terminal differentiation of BCL-1 cells. We also wished to determine if repression of c-myc is sufficient to drive differentiation in this system. Our strategy was to express an inducible dominant negative form of c-Myc. The dominant negative c-Myc (MycDN) contained a deletion in the transactivation domain (amino acids 103 to 143) but retained the basic helix-loop-helix/zipper domains so that it could still associate with Max and bind DNA. Its dominant negative activity has been established in several previous studies, in which it was used to interfere with the function of endogenous c-Myc (9, 20, 40, 63). We used an inducible system in which dominant negative c-Myc was fused to a mutated estrogen receptor (MycDN-ER) and transcribed from the cytomegalovirus promoter. Thus, expression was constitutive and activity could be induced by treatment with the synthetic ligand 4-OHT (15, 40). Immunoblotting revealed that the MycDN-ER fusion protein was present in two lines chosen for study (MycDN-ER-BCL-1 #4 and #6) (Fig. 6A).

In other systems, the induction of MycDN-ER by 4-OHT has been verified by demonstrating a decreased percentage of cells in S phase (40). Therefore, we determined the cell cycle distribution of MycDN-ER-BCL-1 lines before and after induction of MycDN-ER with 500 nM 4-OHT (Fig. 6B). There was only a slight change in the percentage of cells in S phase (45% versus 53%) in a control transfectant after 4-OHT treatment (compare dotted bar to open bar). In contrast, the percentage of cells in S phase in MycDN-ER-BCL-1 transfectants after 4-OHT treatment declined consistently (from 46% to 22% in MycDN-ER #4 and from 52% to 39% in MycDN-ER #6). This shows that MycDN activity is inducible by 4-OHT in these lines and also shows that endogenous c-Myc appears to be more fully inhibited in line #4 than in line #6. We also determined the cell cycle status of these lines following cytokine treatment. Both of the MycDN-ER-BCL-1 lines and the control transfectant were growth arrested after IL-2 and IL-5 treatment (compare solid bar to open bar), indicating that the

FIG. 3—Continued.

transfectants retained the ability to respond normally to cytokine treatment.

These transfectants were then analyzed to determine if inhibition of c-Myc was sufficient to drive differentiation to a

plasma cell phenotype. MycDN-ER-BCL-1 cells were treated with 4-OHT or cytokine for 3 days and then analyzed for markers of the plasma cell phenotype. IgM secretion was analyzed by ELISA, and Fig. 7A shows that in both MycDN-ER-

TABLE 1. Phenotypic changes associated with cytokine-induced differentiation^a

Cells	IL-2 + IL-5 treatment	% of cells in S phase	% of cells expressing Syndecan-1	IgM secretion (ng/ml)
BCL-1	No	43	2	90
	Yes	16	24	930
Myc-BCL-1	No	40	3	38
	Yes	44	7	77
Cyclin E-BCL-1	No	36	3	55
	Yes	39	9	125

^a Parental BCL-1, Myc-BCL-1 (#5), and cyclin E-BCL-1 (#7) cells in log phase (2×10^5) were subjected to differentiation by treatment with recombinant IL-2 and IL-5. After 3 days of cytokine treatment, cells were subjected to cell cycle analysis and surface Syndecan-1 staining, and cell supernatants were used for ELISA analysis as described in the text. Representative data from one experiment are shown.

BCL-1 cells (#4 and #6), activation of MycDN-ER by 4-OHT did not induce IgM secretion, although they responded normally to cytokine treatment. Surface Syndecan-1 levels were also measured: in both MycDN-ER-BCL-1 cells and a control transfectant, Syndecan-1 was induced after IL-2 and IL-5 treatment (Fig. 7B, dashed line). However, 4-OHT alone treatment did not induce Syndecan-1 expression in either MycDN-ER #4 or #6 (compare dark line to dashed line). These results suggest that inhibition of c-Myc and the cessation of cell cycle progression that accompanies it are not sufficient to induce BCL-1 cell differentiation.

It remained a possibility that induction of MycDN did not inhibit cell cycle progression sufficiently to trigger terminal differentiation. Therefore, we sought to arrest BCL-1 cell cycle progression using LOV, which causes growth arrest at G₁ (56) and is sufficient to induce differentiation of human monocytic Mono Mac 6 cells (72). We treated BCL-1 cells with different concentrations of LOV (within a dose range that did not cause cell death) and analyzed the effect of LOV on cell cycle progression as well as on BCL-1 cell differentiation. The percentage of cells in S phase decreased from 36% to 12% after 3 days of 3 μ M LOV treatment, compared to 17% in S phase following cytokine treatment. However, inhibition of cell cycle progression by LOV was not sufficient to cause BCL-1 cells to secrete IgM or to express Syndecan-1 on their surface (not shown), further indicating that inhibition of the cell cycle is not sufficient to drive terminal differentiation of BCL-1 cells.

FIG. 5. c-Myc is downregulated in cyclin E-BCL-1 cells after IL-2 and IL-5 treatment. Log-phase control and cyclin E-BCL-1 #4, #7, and #9 cell lines (2×10^5) were either left untreated or treated for differentiation for 3 days, and cell lysates were harvested for Western blotting analysis as described in Materials and Methods. Protein (10 μ g) was separated by SDS-8% PAGE, and c-Myc and β -actin levels were revealed by probing with specific antibodies. Numbers represent the fold decrease in c-Myc after normalization to β -actin.

DISCUSSION

The data presented here reveal several important aspects of terminal B-cell development in the context of the BCL-1 cell model. First, reduction of c-Myc is necessary for plasma cell differentiation, since constitutive expression of c-Myc blocks differentiation. This establishes the functional importance of c-myc as a target of Blimp-1-dependent repression. Second, continued proliferation is sufficient to block plasma cell differentiation, suggesting that cell proliferation is the aspect of c-Myc function that must be repressed to allow differentiation. Finally, neither blocking of c-Myc activity nor cessation of proliferation is sufficient to trigger terminal differentiation. This leads to the important conclusion that, in addition to c-myc, Blimp-1 must regulate other target genes in order to trigger plasma cell differentiation.

Dissecting the mechanism(s) by which Blimp-1 acts as a master regulator of plasma cell differentiation. Expression of Blimp-1 is sufficient to drive terminal differentiation of BCL-1 cells into plasma-like cells, providing an important experimental system for elucidating the changes in gene expression required for this event (39, 71). The c-myc gene was previously identified as a Blimp-1 target gene, and c-myc repression in BCL-1 correlates with cell cycle arrest (39) (Fig. 2A and B). The data presented here establish the functional relevance of c-myc repression, since enforced expression of c-Myc blocks the ability of these cells to differentiate (Fig. 2). It is interesting to note in this context that *Blimp-1* mRNA was induced normally by cytokine treatment of BCL-1 clones expressing constitutive c-Myc (Fig. 4). Therefore, Blimp-1-dependent regula-

FIG. 4. Blimp-1 mRNA is induced in Myc-BCL-1 and cyclin E-BCL-1 cells after cytokine treatment. Total RNA (10 μ g) from cells (parental BCL-1 cells, Myc-BCL-1 #5, and cyclin E-BCL-1 #7) treated with IL-2 and IL-5 for 0, 2, 10, 24, and 48 h was subjected to RNase protection analysis using [α -³²P]UTP-labeled riboprobes specific for mouse Blimp-1 and GAPDH. Numbers represent the fold induction of *Blimp-1* mRNA after normalization to the level of *GAPDH* mRNA.

FIG. 6. Induction of dominant negative c-Myc activity by 4-OHT in MycDN-ER-BCL-1 cells. (A) Immunoblots for the detection of dominant negative Myc-ER fusion protein. The position of the 83-kDa size marker is shown on the left. N.S., nonspecific band. (B) Cell cycle status of 2×10^5 cells (control and MycDN-ER #4 and #6) left untreated (open bar) or treated with cytokine (solid bar) or 500 nM 4-OHT (dotted bar) for 3 days. Results show the percentage of cells in S phase determined by FACS after BrdU and propidium iodide staining. Experiments were done twice, and one set of results is shown.

tion of other possible target genes is not sufficient to overcome the inhibition by c-Myc.

The data in Fig. 7 show, however, that dominant negative c-Myc was not sufficient to trigger differentiation of BCL-1 cells. In contrast, inhibition of c-Myc activity can induce differentiation of some nonlymphoid cell lines (23, 25, 73). Also, overexpression of the cyclin-dependent kinase inhibitor p18^{INK4c} in human lymphoblastoid SKW cells caused differentiation (47). It is likely that differences in the developmental stage may account for the difference in the response of SKW and BCL-1 cells to cessation of the cell cycle.

It is formally possible that c-Myc activity was not sufficiently inhibited in any of our MycDN-ER-BCL-1 lines to induce differentiation. However, we also blocked BCL-1 cell proliferation with LOV, since our data showed that c-Myc's proliferative activity is critical for plasma cell differentiation (Fig. 3). Even when more complete cessation of cycle was achieved with LOV, this was not sufficient to induce BCL-1 differentiation. Thus, neither expression of MycDN-ER nor cessation of proliferation is sufficient to trigger terminal differentiation. These data, in conjunction with the observation that enforced proliferation blocks terminal differentiation, support the conclusion that repression of c-myc transcription is necessary but not sufficient to drive BCL-1 differentiation. We suggest that reg-

ulation of other target genes in addition to c-myc is required during the Blimp-1 program of terminal differentiation.

If other Blimp-1 target genes are important during BCL-1 differentiation, what might those target genes be and how might Blimp-1 affect their transcription? It seems most likely that Blimp-1 target genes will be repressed, since there is ample evidence that Blimp-1 is a transcriptional repressor. Its human homologue, PRDI-BF1, also functions as a transcriptional repressor of the *interferon-β* gene (27, 31). Blimp-1 represses transcription of a heterologous promoter in a GAL4 fusion assay (74). Blimp-1/PRDI-BF1 mediates transcriptional repression via the association with both hGroucho (58) and histone deacetylase (74) proteins. However, these data do not rule out the possibility that Blimp-1 might also activate transcription in the context of a currently unidentified target gene.

Several genes, including *BCL-6* (48), *CIITA* (68), *BSAP* (5), *A1* (32), and *CD23* (61), are known to be transcriptionally repressed upon plasma cell differentiation and are good candidates to be direct targets of Blimp-1. Indeed, we have recently shown that the P3 promoter of the *CIITA* gene, encoding a coactivator required for *class II MHC*, *Ii*, and *DM* gene transcription, is directly repressed by Blimp-1 (Piskurich et al., submitted). In addition, there are likely to be Blimp-1 target genes as yet unidentified. Our current model for the roles of

A. **B.**

FIG. 7. Cell cycle arrest by dominant negative c-Myc does not cause differentiation of BCL-1 cells. (A) IgM secretion in control and MycDNER-BCL-1 #4 and #6 cells after 3 days of cytokine or 4-OHT treatment. The 2×10^5 cells were left untreated or treated with recombinant IL-2 and IL-5 or 500 nM 4-OHT for 3 days, and supernatants were collected and assayed by ELISA for IgM levels. Results show the averages from three independent determinations. Standard deviations are also shown. (B) Cell surface Syndecan-1 staining profiles analyzed by FACS of control (left panel), MycDNER-BCL-1 #4 (middle panel), and MycDNER-BCL-1 #6 (right panel) cells following cytokine or 4-OHT treatment. The cells were left untreated (fine line) or treated with recombinant IL-2 and IL-5 (dashed line) or 500 nM 4-OHT (thick line) for 3 days, and surface marker staining using anti-Syndecan-1 antibody was performed. Experiments were done three times, giving similar results.

FIG. 8. Model for the roles of Blimp-1 and c-Myc in B-cell terminal differentiation. II°, secondary. ODC, ornithine decarboxylase. MHCII, major histocompatibility complex II.

c-myc and other genes in Blimp-1-dependent plasma cell differentiation is summarized in Fig. 8.

Role of c-Myc in terminal B-cell differentiation. Inhibition of plasma cell differentiation by constitutive expression of c-Myc (Fig. 2) is consistent with similar observations in other cell lineages in which ectopic c-Myc blocks differentiation (13, 25, 26, 35, 50). Furthermore, mice harboring an E μ -*c-myc* transgene develop B-cell lymphomas (1), display abnormal apoptosis (51), and have little evidence of terminal differentiation (67), consistent with the requirement for *c-myc* repression during plasma cell development.

The mechanism(s) by which c-Myc blocks differentiation is not well understood. Our data show that proliferation is sufficient to inhibit BCL-1 differentiation, since the effect of constitutive c-Myc can be mimicked by constitutive cyclin E (Fig. 3). In the cyclin E-BCL-1 lines, *Blimp-1* mRNA and c-Myc are normally induced or repressed, respectively, in response to cytokine treatment, so that other Blimp-1- or c-Myc-dependent target genes are presumably regulated normally. However, this is not sufficient to overcome the inhibition of differentiation caused by continued proliferation. Thus, c-Myc target genes that drive proliferation are the ones that must be correctly regulated to allow terminal B-cell differentiation. These target genes may regulate cell cycling directly or may regulate cell growth and metabolism or both (reviewed in reference 14). Cell cycle regulators Gadd45, Cdc25A, and cyclins A and E are induced by c-Myc, and c-Myc also regulates the activity of cyclin E-Cdk2 (43, 70). In addition, c-Myc target genes involved in metabolism and growth control, such as the genes for carbamoyl-phosphate synthase-aspartate carbamoyl transferase-dihydroorotase, ornithine decarboxylase, dihydrofolate reductase, thymidine kinase, lactate dehydrogenase A, H-ferritin, iron regulatory protein 2, eIF4E, and eIF2a, may be

critical for cell proliferation (14, 28, 29). It will be interesting to establish stable BCL-1 lines expressing individual c-Myc target genes to examine their effect on differentiation. Similar studies in M1 myeloid leukemia cells indicated that ectopic expression of ornithine decarboxylase could not mimic c-Myc's effect on differentiation (25).

Results obtained with any cell line need to be interpreted cautiously with respect to extrapolation to mechanisms in normal cells. However, BCL-1 cells have provided an accurate model for studying many aspects of B-cell biology in the past. In addition, we have shown that induction of *Blimp-1* mRNA, repression of *c-myc* mRNA, and cessation of cell cycling also occur upon lipopolysaccharide treatment of primary splenic B cells (3). We have also found that Blimp-1 is expressed in vivo in murine and human plasma cells (3a). Thus, the observations made in the BCL-1 cell model are likely to have important implications for terminal differentiation of B cells in vivo.

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