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B Lymphocyte Commitment Program Is Driven by the Proto-Oncogene c-myc

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c-Myc, a member of the Myc family of transcription factors, is involved in numerous biological functions including the regulation of cell proliferation, differentiation, and apoptosis in various cell types. Of all of its functions, the role of c-Myc in cell differentiation is one of the least understood. We addressed the role of c-Myc in B lymphocyte differentiation. We found that c-Myc is essential from early stages of B lymphocyte differentiation in vivo and regulates this process by providing B cell identity via direct transcriptional regulation of the ebf-1 gene. Our data show that c-Myc influences early B lymphocyte differentiation by promoting activation of B cell identity genes, thus linking transcription factor to the EBF-1/Pax-5 pathway. The Journal of Immunology, 2011, 186: 6726–6736.

The generation of mature B lymphocytes from early lymphoid progenitors in the bone marrow (BM) is a well-defined process characterized by several cell stages in which a number of transcription factors play a prominent role (1). In BM, pro-B cells (B220+c-Kit+) begin sequential rearrangement of the IgH locus gene segments (V, D, and J) and differentiate into pre-B lymphocytes (B220+CD25+). Productive rearrangement of the H chain locus triggers L chain rearrangement and cell surface expression of both H and L chains (IgM). This process gives rise to immature B cells (B220+IgM+) that migrate from the BM to secondary lymphoid organs to generate mature B lymphocytes (2).

The transcription factors E2A, EBF-1, and Pax-5 have a critical function in early B cell commitment and differentiation (3). Mouse models of gene inactivation have shown the central role of these transcription factors in these processes. Gene inactivation of tcf/e2a (4, 5) or ebf-1 (6) in mice leads to an early block in B cell differentiation before the onset of IgH rearrangement. Both factors appear to work in synergy to activate B cell-specific B lymphocyte genes, conferring B cell identity on early lymphoid precursors (7). E2A-deficient pro-B cells are rescued by ectopic expression of EBF-1 in vitro but not by Pax-5 (8). Inactivation of pax-5 in mice causes a block in early B cell differentiation (9) and impaired V_{H}D_{H}J_{H} rearrangement (10). Pax-5 regulates the expression of the B cell-specific genes cd19 (11), btk (12), and cd79a (13) and represses the expression of genes incompatible with B lymphocyte differentiation (14). Ectopic Pax-5 expression is not capable of promoting B cell differentiation in ebf-1−/− progenitors. EBF-1 induces pax-5 gene expression and activates the B cell transcriptional program (15). Taken together, a model has been proposed in which E2A, EBF-1, and Pax-5 act sequentially to promote commitment to B cell fate (7, 15).

The Myc proteins (N-, L-, and c-Myc) are members of a basic region/helix-loop-helix/leucine zipper transcription factor family and are involved in many biological functions. All of the Myc proteins heterodimerize with Max and bind to specific sites on the DNA (E-boxes) to regulate their target genes (16); of all of the Myc proteins, c-Myc is probably the best studied. In humans and mice, c-Myc deregulation is well established as a primary cause of some cancers. It is estimated that the c-myc proto-oncogene is activated in 20% of all human cancers (17). It is expressed in many cell types as well as in early BM progenitors and during B lymphocyte differentiation (18).

Accumulated in vivo and in vitro evidence shows that c-Myc participates in regulating cell proliferation, differentiation, and apoptosis in many cell settings, including B lymphocytes (16). During cell cycling, c-Myc promotes G0/G1-S transition by activating genes that encode proteins of the cyclin/cyclin-dependent kinase complexes and by repressing cell cycle inhibitors such as p21 or p27 in numerous cell types (19). In murine B cell lymphoma lines, apoptosis induced through the BCR correlates with the inhibition of c-myc expression (20). In mice, mature B lymphocytes lacking c-Myc show impaired proliferation and elevated levels of the cell cycle inhibitor p27 as well as greater resistance to apoptosis (21, 22). c-Myc overexpression in transgenic mouse B cells leads to rapid lymphoma development and mouse death (23).

Despite numerous studies of c-Myc, little is known about its function in B cell differentiation (24). c-Myc downregulation is associated with cell cycle arrest and terminal differentiation in...
B lymphocytes and myeloid cells (25–27). Here, we address the role of c-Myc in early B lymphocyte differentiation, using several conditional mouse models. Our data provide evidence that c-Myc influences B lymphocyte differentiation through the EBF-1/Pax-5 pathway, thus activating B cell identity genes. Finally, our results place c-Myc in the context of transcription factors required for B lymphocyte differentiation.

Materials and Methods

Mice and genotyping

Generation of c-myc^fl^/^-mex^-cre^+ mice was described (28). To generate c-myc^fl^/^-mbl^-cre^+ mice, c-myc^fl^/^- cre^+ mice were bred with mbl^-cre^+ mice (29), and progeny were crossed to yield homozygous (c-myc^fl^/^-mbl^-cre^+/^-) and control mice (c-myc^fl^/^-mbl^-cre^+/- or c-myc^fl^/^-mbl^-cre^-/-). c-myc^fl^/^-mex^-cre^+ or c-myc^fl^/^-mex^-cre^-/- mice were bred with ik^nest^-/- mice (30) to generate ik^nest^-, c-myc^fl^/^-mex^-cre^+ or ik^nest^-, c-myc^fl^/^-mex^-cre^-/- mice respectively. Progeny were crossed to generate homozygous (ik^nest^-/-; c-myc^fl^/^-mex^-cre^+ or ik^nest^-/-; c-myc^fl^/^-mex^-cre^-/-) mice. Mice were genotyped using a PCR-based analysis of tail genomic DNA (28). Primers 5'-ACC TCT GAT GAA GTC TAG-3' (here-DIR) and 5'-AGG AAG AAC-3' (here-REV) were used to amplify mbl-cre (+) DNA (AGG AAG AAC-3'). mbl-wt alleles (mbl1 and mbl2). The knock-in allele (ik^nest^-/-) was identified as described (30). c-myc^fl^/^-mex^-cre^+ or c-myc^fl^/^-mbl^-cre^+ mice were bred with rosa26^GFP^-/- mice to generate c-myc^fl^/^-mex^-cre^+;rosa26^GFP^-/- or c-myc^fl^/^-mbl^-cre^+;rosa26^GFP^-/- mice, respectively. The Rosa26 allele was genotyped as described (31).

Polyinosinic-polycytidylic acid injections

To induce c-myc deletion in c-myc^fl^/^-mex^-cre^+ and ik^nest^-/-c-myc^fl^/^-mex^-cre^+ mice, 4- to 6-wk-old animals received three i.p. injections of polynucleotides saline (pLPC; Amersham Biosciences) (200 µg each) at 2-day intervals and were analyzed 3 d after the last dose.

Flow cytometry analysis and cell sorting

For cell sorting or flow cytometry analysis, BM B lymphocytes were purified (FACS Coulter cell sorter) and/or analyzed as pro-B (Ly-6c^+;NK1.1^-; B220^+;CD25^-; IgM^-;DX5^-;CD25^-;IgM^-;DX5^-) and pre-B cells (Ly-6c^-;NK1.1^-; B220^-;CD25^+;IgM^+;DX5^+;NK1.1^-). Purity >97% was verified by flow cytometry reanalysis. Anti-B220 Abs were conjugated either with PE-Cy7 (eBioscience), FITC, or allophycocyanin (Becton Dickinson). Anti-IgM Abs (Southern Bio-technology Associates) were conjugated with either PE or biotin. Allo- phosphorycin (eBioscience) or streptavidin (BD Pharmingen) or PE-Texas Red–streptavidin (Immunotech) was used to conjugate with biotin. FITC- or biotin-conjugated anti–Ly-6c (Becton Dickinson), anti-NK1.1^-/-, and anti-DX5- (both from BD Pharmingen) Abs were used in a dunn channel to remove contaminating NK and dendritic cells.

Brdu labeling

Brdu incorporation was assessed 2 h after a single i.v. Brdu injection (1 mg/15 g body weight; Sigma-Aldrich). Pro-B (Ly-6c^+; NK1.1^-; DX5^-; B220^+;Kit^+IgM^-) and pre-B cells (Ly-6c^-; NK1.1^-; DX5^-; B220^-; CD25^+IgM^-) from 16- to 24-wk-old mice were sorted from BM, and Brdu incorporation was measured using FITC- or PE-anti–Brdu MAb (BD Biosciences) or propidium iodide, following standard protocols. The same protocol for Brdu incorporation was used for sorted B220^-IgM^- and immature cells from ms-fl^/^- and fl^/^- control mice.

Retrovirus production and transduction

Flat-E cells were seeded (2 x 10^6 cells) in 6-cm plates 18-24 h before transfection with MIG-R1 or MIG-EFBI plasmids (3 µg) in the presence of FuGene 6 reagent (Roche Diagnostics). Retroviral supernatants were collected 48 h after transfection and filtered through a 45-µm filter, and 1 µM 1-glutamine and supplemented with recombinant murine stem cell factor (50 ng/ml), recombinant murine IL-6 (5 ng/ml), and murine LIF (10^3 U/ml). Stimulated Lin^- cells were transduced by spin infection after 24 h of culture. Cells were resupended in 1 ml fresh retroviral supernatant, supplemented with 10 µg/ml polybrene (Sigma-Aldrich) and cytokines as above. Cells were centrifuged (1136 x g, 90 min, 32°C) and incubated (3–4 h). Medium was replaced with IMDM containing 2% heat-inactivated FBS, 1 mM L-glutamine, 1 mM penicillin/streptomycin, 0.03% w/v Pristane (Sigma-Aldrich), and 50 mM 2-ME and supplemented with recombinant stem cell factor (10 ng/ml), rFlt3L (10 ng/ml), and rIL-7 (10 ng/ml). Transduction efficiency was monitored by flow cytometry at 48 or 72 h postinfection.

VDJ recombination analysis

Genomic PCR amplification of Ig genes was performed with Vq-specific primers from Fuxa et al. (32); DJ primers were from Ehlrich et al. (33). GAPDH was used as a loading control. PCR products were electrophoresed, and the bands were quantified with ImageJ software. For VDJ sequencing, PCR fragments were amplified with FastStart High Fidelity polymerase (Roche), and the Vq558-Ig-J3 band was cloned in PCR2-TOPO. Sequences were analyzed with the IGMT Junctions Analysis program.

Gene expression analysis

For quantitative PCR (qPCR) analysis, 2.5 µl cDNA (10-fold dilution series) was mixed with primers and SYBR Green PCR Master Mix (BD Biosciences). All of the oligonucleotides were designed to yield 70- to 130-bp PCR fragments. Oligonucleotides for c-myc and β-actin were as described (34). Primers for cd19 were CD19 sense (5'-AGTACCGAGATGT-GCTCTCC-3') and antisense (5'-GAGTCTTGAATGGGTAGGGT-3') for cd19, E2A s (5'-ATAACGAGGAGTGCCCACT-3') and E2A as (5'-CTCAAGGTCACACTCTGTAG-3') for e2faE1, s (5'-CTATGTTG-GCCCTACGACT-3') and E2F-1 as (5'-CATGATCTGCTGCTCTGATAGGA-3') for e2F-1, Flt3 (5'-CAGCCCGACTTTGATTTACA-3') and Flt3 as (5'-GGATCTGGCTCTGATAGGA-3') for flt3, Ikaro s (5'-TTGTTG-GGATCTGGCTCTGATAGGA-3') and Ikaro s (5'-GAGTCTTGAATGGGTAGGGT-3') for ikaro, Lef-1 s (5'-TCAGTCTGACCAAGGAGGAGG-3') and Lef-1 as (5'-CCCTGAGCTCTGGCTGTAG-3') for liff1, Pu-1 s (5'-GGGTATCCACACTTGAGGA-3') and Pu-1 as (5'-GAGTCTGAGGCAAAGAGGGAGG-3') for pu-1, Lef-1 s (5'-CCCTGAGCTCTGGCTGTAG-3') for Lef-1, Pu-1 s (5'-GGTGGATCTGGCTCTGATAGGA-3') and Pu-1 as (5'-GAGTCTGAGGCAAAGAGGGAGG-3') for pu-1, and Lef-1 as (5'-AAATTCGTCAAGGAGGAGGAGG-3') for liff1. For the remainder of the genes, predesigned Applied Biosystems Micro Fluidic cards were used. Each gene was analyzed in triplicate. cDNA samples and reagents and reagents were applied on an Applied Biosystems Prism 7900HT. Data were analyzed with SDS2.2 sequence detection systems.

Luciferase activity assay

The murine elf-1 α promoter was amplified by PCR using the primers Elf-1οs (5'-TAATAGGCGCGCAATCTGCCC-3') and Elf-1οx (5'-GCTGAA-GAATCTGCGCAAGTGT-3'), cloned into the EcoRI site of the TOPO 2.1 vector (Invitrogen), and subcloned into the NheIBglII site of the plg3L-Control vector (Promega) upstream of the luciferase gene to generate the vector pGL3-Efβ-1α. All of the constructs were sequenced. For luciferase assays, HEK 293T cells were cultured in 24-well plates and cotransfected with 500 ng pGL3-Efβ-1x or pGL3-Control vector and increasing amounts of pRyr-ires-GFP-c-Myc expression vector. Renilla luciferase activity was used for normalization. At 48 h after transfection, firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega).

Chromatin immunoprecipitation assays

Experiments were performed following the protocol of the chromatin immunoprecipitation (ChIP) assay kit (Active Motif). 1×10^5 cells were cross-linked with formaldehyde (1% final concentration) and incubated (room temperature, 20 min). Rabbit polyclonal anti-c-Myc N262 Ab (sc-764; Santa Cruz Biotechnology) or preimmune serum was used to precipitate chromatin from 2 x 10^6 cells. Immunoprecipitated DNA and input samples were analyzed with a SYBR Green RT-PCR kit (Applied Biosystems), and percentage enrichment relative to the amount of input chromatin was determined as (CP input/CP input + input) x 100. Abs: primers flanking E-box5 were: E5-FW (5'-GGTAGAGAACG-3') and EB5-REV (5'-ACTCGCAGGA-GGTAGAGAG-3').

EMSA

Assays were performed with labeled double-stranded oligonucleotides encompassing E-boxes from the elfb and elf-1 α promoters. pcDNA3-c-
Myeloid and pcdNA3-Max were in vitro-translated using TNT-coupled reticulocyte lysate systems (Promega). Binding reactions between in vitro-translated proteins and labeled probes (1 ng) were performed as described (35), except that 0.25× Tris-borate-EDTA was used. Unlabeled oligonucleotide competitors (100 ng) and either 1 μg anti-c-Myc (sc-7646x) or 1 μg anti-c-Myc (sc-517x) Abs (both from Santa Cruz Biotechnology) were used. Double-stranded oligonucleotides used were: DHFR-F-1wt (5’-GGCCGGACACCGTGCTTCC-3’ and 5’-AGAGAGGCACGGTGCTG-3’), EB5-wt (5’-GGCTCTACCCGTATCTGACGCTACTGAGT-3’ and 5’-GAGACTGCACTCAAGGGATGTTAGGA-3’), EB5-mut (5’-GGCTCTACCCGTATCTGACGCTACTGAGT-3’ and 5’-GAGACTGCACTCAAGGGATGTTAGGA-3’), EB505-wt (5’-GCTTTTCCTACGTCATACGGGTGTTAGGA-3’ and 5’-GTCCAACCTGCTTGATGCTGAGGAAA-3’), EB505-mut (5’-GCTTTTCCTACGTCATACGGGTGTTAGGA-3’ and 5’-GTCCAACCTGCTTGATGCTGAGGAAA-3’). Complementary oligonucleotides were mixed at an equimolar ratio in 10 mM Tris (pH 7.5)/50 mM NaCl, heated to 65°C, and annealed by slow cooling to room temperature. Double-stranded oligonucleotides (100 ng) were labeled by a Klenow fill-in reaction.

Results

c-Myc is necessary for B lymphocyte differentiation

To study the role of c-Myc in B cell differentiation, we conditionally inactivated the c-myc gene in developing B lymphocytes by breeding the fl/fl (21) conditional mouse with mb1-cre (31) knock-in (29), mx-cre transgenic (36), and rosa26rstopfp reporter mice (31). In mb1-fl/fl mice, the mb1-cre allele is expressed from the earliest stage of B lymphocyte differentiation (29). In mx-fl/fl mice, Cre recombinase is induced after injection of IFN or pIpC and efficiently deletes c-myc in BM (28). In addition, in gfp-mb1-fl/fl and in gfp-mx-fl/fl mice, the rosa26rstopfp allele expresses GFP after activation of Cre recombinase (31) (Supplemental Fig. 1A). To determine whether c-myc inactivation affects B cell differentiation, we used flow cytometry to analyze the B cell populations in the BM and spleen of mb1-fl/fl mice (37) (38). Deletion of the c-myc gene at early differentiation stages led to a developmental defect at the pro- to pre-B cell transition in BM of mb1-fl/fl and gfp-mb1-fl/fl mice (Fig. 1A, 1B, Supplemental Fig. 1B). Analysis of Hardy fractions in these mice also revealed a developmental defect and a decrease in absolute numbers in Fractions B, C, and D (large pre-B cells), which is consistent with the time in which deletion of c-myc occurs (Fig. 1C, 1D). Similar results were obtained when CD19 was used as a B cell marker (Supplemental Fig. 2, A–D). The absolute number of B lymphocytes (B220+) in mb1-fl/fl mouse BM was 4-fold lower than those of controls (0.4 × 10^6 versus 1.8 × 10^6) (Fig. 1E). mb1-fl/fl spleens showed a 54-fold decrease (0.2 × 10^6 versus 8.1 × 10^5) in the number of mature B lymphocytes (B220+IgM+) compared with those of controls (Fig. 1E). Genomic PCR analysis of these cells confirmed c-myc deletion (Supplemental Fig. 1D, 1E). In vitro cultures of gfp-mb1-fl/fl mouse BM cells did not generate IgM+ B lymphocytes, suggesting that the absence of c-Myc in mb1-fl/fl and gfp-mb1-fl/fl mouse BM prevents the generation of mature B cells in spleen (Supplemental Fig. 3). To test the apoptotic status of pro- and pre-B cells in mb1-fl/fl mouse BM, we monitored apoptosis by flow cytometry using annexin V and found a 2-fold increase (2.8 versus 6.1%) in pro-B cells and a 25-fold increase in pre-B cells (0.9 versus 23.1%) from mb1-fl/fl mice compared with those from controls (Fig. 2A). c-Myc appears to be necessary for pre-B cell survival in mb1-fl/fl mice and thus to be required from early stages of B cell differentiation.

To study the c-Myc requirement at later stages of B cell development, we injected pIpC to induce c-myc deletion in mx-fl/fl mouse BM. Cell proliferation in developing c-Myc–deficient B lymphocytes

To determine whether c-Myc inactivation affected cell proliferation, we monitored in vivo BrdU incorporation in mb1-fl/fl and pIpC-injected mx-fl/fl mice. Sorted pro- and pre-B lymphocytes from mb1-fl/fl mice still retain some capacity to proliferate compared with that in controls (54.5 versus 25.4% in pro-B and 51.5 versus 6.1% in pre-B BrdU+ cells) by flow cytometry analysis (Fig. 3A). The mx-fl/fl mouse pro- and pre-B cells showed similar proliferative capacities (Fig. 3B). We did not observe significant differences in proliferation in immature lymphocytes from mx-fl/fl mice (Fig. 3B). We concluded that developing B lymphocytes have a reduced capacity to proliferate in the absence of c-Myc in vivo.

Reduced levels of V(DJ) recombination in c-Myc–deficient B lymphocytes

Sequential rearrangement of the V(DJ) gene segments that encode the BCR is linked intrinsically to B lymphocyte differentiation (2). To determine whether the developmental defect in c-Myc–deficient B lymphocytes was characterized by a lack of or impaired Ig gene recombination, we analyzed V(DJ) rearrangement by genomic PCR in sorted pro- and pre-B cells. We did not observe significant differences in D1-J5 and V_{Hproximal} and V_{Hdistal}-to-D4J4 rearrangements in purified pro-B cells from mb1-fl/fl mouse BM compared with those of control cells (Fig. 3C, upper panel, and Fig. 3D, p > 0.05). In contrast, purified pre-B cells from mutant mice showed a slight decrease in V_{Hproximal} (1.9-fold) and V_{Hdistal}-to-D4J4 (2.4-fold) rearrangements compared with those of control mice (Fig. 3C, lower panel, and Fig. 3D). These differences between pro- and pre-B cells likely reflect the time in which c-myc deletion occurs. Pro-B cells are undergoing c-myc deletion, and pre-B cells have completed it. To see whether these recombination events were normal, we sequenced some V(DJ) rearrangements from mb1-fl/fl and mb1-fl/+ mouse BM cells and observed no apparent differences between both populations (Supplemental Table I).

Gene expression in c-Myc–deficient B lymphocytes

To define the molecular mechanism by which c-Myc acts on B lymphocyte differentiation, we analyzed gene expression of key transcription factors involved in this process. qPCR showed that cmyc, ebf1, ikaros, and pax-5 expression was downregulated slightly in sorted c-Myc–deficient pro-B cells, and this effect was more dramatic in pre-B cells. This is likely due to the timing in which c-myc deletion occurs in mb1-cre mice. (Fig. 4A). Inter-
Interestingly, *tcfe2a<sup>−/−</sup>, ebf-1<sup>−/−</sup>, and *pax-5<sup>−/−</sup> mice have a block at early stages of B cell development as well as impaired V(D)J recombination (4, 6, 9). EBF-1 shares some target genes with Pax-5 and transcriptionally regulates its expression providing B cell identity (7). Expression of Pax-5 target genes such as *cd19* (11) was reduced and that of the repressed target gene *flt3* (40) was increased in pro- and pre-B cells from *mb1-fl/fl* mice compared with those of the controls (Fig. 4A). Flow cytometry showed fewer pro- and pre-B cells with surface expression of CD19 (7.2 versus 68.4%) or pre-BCR (0.1 versus 0.3%) in *mb1-fl/fl* mouse BM compared with those of controls (Fig. 4B, 4C). Interestingly, *flt3* gene expression was highly increased in c-Myc–deficient pre-B cells (36-fold) compared with that of control cells, which is higher than that described for *pax-5<sup>−/−</sup> B cells (14). This probably reflects the need for additional factors under the control of c-Myc, other than Pax-5, which is required for normal regulation of the *flt3* promoter. In contrast, the *cd19* promoter, under the tight control of Pax-5, is more sensitive to small variations of gene expression of this transcription factor (Fig. 4C).

Consistent with these results, gene expression of the Pax-5 target genes *blik* (12), *cd79a*, *cd79b*, and *n-myc* (41) was reduced in B220<sup>+</sup>IgM<sup>−</sup> cells (pro- and pre-B cells) from *mb1-fl/fl* mice (Supplemental Fig. 4A). Similarly, pro- and pre-B cells from *gfp-mx-fl/fl* mice showed reduced *cd79b*, *cd79a*, and *pax-5* expression (Supplemental Fig. 4B). To rule out contamination with non-B cells, we tested c-my and *flt3* expression in pro- and pre-B cells purified using a mixture of Abs to pro-B (Ly-6c<sup>−</sup>NK1.1<sup>−</sup>DX5<sup>−</sup>B220<sup>c-Kit<sup>−/−</sup>IgM<sup>−</sup>)) and pre-B cells (Ly-6c<sup>−</sup>NK1.1<sup>−</sup>DX5<sup>−</sup>B220<sup>c-Kit<sup>−/−</sup>IgM<sup>−</sup>)).
CD25^IgM^- (Supplemental Fig. 4C, 4D). To test whether decreased pax-5 expression promoted transdifferentiation of c-Myc-deficient B cells into T cells (42), we cultured pro- and pre-B cells with the OP9-DL1 cell line (43). Genomic PCR analysis indicated no TCR recombination in cultures of c-Myc-deficient B cells from gfp-mb1-fl/fl mouse BM (Supplemental Fig. 5).

**EBF-1 is a transcriptional target of c-Myc**

ebf-1 gene expression is controlled by two promoters, α and β, which are regulated differentially in B cells (44). We identified two conserved c-Myc binding sites (E-boxes) in human and mouse, upstream of or within the ebf-1 α promoter. In reporter assays on HEK 293T fibroblasts, a 1.5-kb genomic region containing the ebf-1 α promoter did not activate the luciferase gene in a c-Myc dose-dependent manner (Supplemental Fig. 5A, 5B). Mutant deletion analysis identified a 0.9-kb region of the ebf-1 α promoter that activated the luciferase reporter in a c-Myc dose-dependent manner in fibroblasts (2-fold) (Fig. 5A, 5B).

To determine whether c-Myc binds to a genomic region containing E-box5, we performed ChIP assays in L1-2 cells (45). Using specific primers that flank E-box5, we observed a 10-fold enrichment by PCR of the DNA fragments immunoprecipitated with a c-Myc-specific Ab compared with that with preimmune serum (Fig. 5D). We used EMSAs to determine whether c-Myc bound specifically to this E-box5; c-Myc bound to oligonucleotides containing E-box5 from the ebf-1 locus. Mutated E-box5 or E-box4 did not compete for c-Myc binding with unmutated E-box5, as determined using anti-c-Myc Ab (Fig. 5E, upper panel). c-Myc binds to an E-box located in a region 5' of the dhfr gene (46). We observed that E-box5 from ebf-1 competed for c-Myc binding with oligonucleotides containing the dhfr E-box. Mutated ebf-1 E-box5 or E-box4 did not compete with the dhfr E-box (Fig. 5E, lower panel). Altogether, these data show that c-
The Journal of Immunology

FIGURE 4. Gene expression in c-Myc-deficient B lymphocytes. A, qPCR of sorted pro- and pre-B cells from mb1-fl/fl and mb1-fl/+ control mouse BM. Each panel shows an independent experiment (mean ± SD for three mutant and three control mice); numbers indicate the x-fold change (2^ΔΔCt). B and C, Flow cytometry analysis of pre-BCR and CD19 surface expression on mb1-fl/fl and mb1-fl/+ control mouse BM lymphocytes. c-myc transgenic (Eu-c-myc) and rag-1^-/- mice were included for comparison. Data represent at least three independent experiments.

Myc directly regulates ebf-1 transcription by binding to the E-box5 in the ebf-1 α-promoter.

In vitro rescue of B cell differentiation in c-Myc-deficient B lymphocytes

To determine whether restoration of EBF-1 expression in c-Myc-deficient B lymphocytes promotes B cell differentiation, we cultured BM progenitors (Lin^-) from mb1-fl/fl and mb1-fl/+ control mice and infected them with a retrovirus expressing EBF-1-GFP or a GFP-Control vector. After 6 days of culture with IL-7, the BM progenitors from mb1-fl/fl mice infected with EBF-1–expressing retrovirus generated c-Myc-deficient B220^CD19^GFP^ cells (Fig. 6A). We did not observe surface expression of IgM in c-Myc-deficient B220^CD19^GFP^ cells infected with EBF-1–expressing retrovirus (data not shown). To see whether EBF-1–induced differentiation in c-Myc-deficient B cells affected V(D)J recombination, we performed genomic PCR on these cells. Genomic DNA was isolated from either sorted B220^CD19^GFP^ or B220^CD19^GFP^ cells infected with either EBF-1 or GFP control retrovirus from mb1-fl/fl and mb1-fl/+ control mice. The B220^CD19^GFP^ population was not generated from mb1-fl/fl mice when infected with control retrovirus (Fig. 6A). We observed an increase in D_{H1}-J_{H1} rearrangements in EBF-1–infected B220^CD19^GFP^ cells compared with those in B220^CD19^GFP^ cells infected with control retrovirus (Fig. 6B). V_{H1}-D_{H1} rearrangements were hardly detected in both populations infected with either retrovirus (Fig. 6B). We concluded from these experiments that EBF-1 promoted B cell differentiation in c-Myc-deficient B cells by inducing CD19 expression and contributing to DJ rearrangements.

To see whether rescue by EBF-1 of B lymphocyte differentiation in c-Myc-deficient B cells affected cell proliferation, B220^CD19^ infected cells from mb1-fl/fl and mb1-fl/+ control mice were stained with propidium iodide. We observed that expression of EBF-1 did not restore the normal capacity to proliferate (39.4 versus 11.8%) in c-Myc-deficient B cells (Fig. 6C).

pax-5 is regulated transcriptionally by EBF-1 and activates B cell-specific genes such as cd19 (11, 44), conferring B cell identity on these cells (7). To test whether ectopic expression of EBF-1 was accompanied by the activation of pax-5 expression, we performed qPCR in sorted B220^CD19^GFP^ cells. c-Myc-deficient B220^CD19^GFP^ cells expressed higher pax-5 levels than control retrovirus-infected c-Myc-deficient B220^CD19^GFP^ cells from the same mice. We did not observe changes in gene expression of n-myc and tce2a (Fig. 6D). We concluded that ectopic expression EBF-1 promotes B cell differentiation by inducing CD19 expression in c-Myc-deficient B lymphocytes.

To test whether Pax-5 expression alone contributed to the rescue of B lymphocyte differentiation in c-Myc-deficient B lymphocytes, we bred mx-fl/fl with ik^neo^+ mouse (30) to generate ik-mx-fl/fl mice. ik^neo^+ mice express pax-5 from the endogenous ikaros promoter upon deletion by Cre recombinase of a stop codon flanked by loxP sites. In ik-mx-fl/fl mice, plpC injection leads to Cre recombinase expression and deletion of c-myc and activation of pax-5 expression from the endogenous ikaros promoter. Attempts to rescue B cell differentiation by expressing pax-5 in ik-mx-fl/fl mice were unsuccessful probably due to the low levels of pax-5 expression in these mice (Supplemental Figs. 7A–C, 8). Flow cytometry analysis of B cell populations in the BM of plpC-injected ik-mx-fl/fl mice showed no significant differences in the number of c-Myc-deficient pro- and pre-B cells and a 5-fold increase in the number of immature B lymphocytes (0.5 × 10^6 versus 0.1 × 10^6) compared with those of mx-fl/fl mice (Fig. 7). We also observed an increase in the number of CD19-expressing pro- and pre-B cells in ik-mx-fl/fl mice (73.1 versus 42.5%) (Fig. 7A). We concluded that Pax-5 contributed to promote B cell differentiation in c-Myc-deficient B lymphocytes.

Discussion

Since the discovery of c-Myc, an extensive scientific literature has addressed its function in various experimental settings (16). The prominent role of c-Myc in the cell cycle and in apoptosis has been the focus of many reports using various cell types, including B lymphocytes. The specific function of this gene in B lymphocyte differentiation nonetheless remains poorly understood, probably due to the lack of mouse models suitable for its...
FIGURE 5. c-Myc transcriptionally regulates ebf-1. A, Luciferase reporter constructs. B and C, c-Myc dose-dependent activation of ebf-1 in HEK 293T and L1-2 cells (B cells) in transient transfection assays. Luciferase activity was normalized with Renilla activity (relative luciferase units, RLU). Mean ± SD for three replicates in one representative experiment. D, ChIP assays in L1-2 B cells. Immunoprecipitation was performed with anti-c-Myc Ab (N262) or preimmune serum (control). Data show the mean of three independent experiments. E, EMSAs. In vitro-translated c-Myc and Max proteins were incubated with oligonucleotides containing E-box5 from ebf-1 (upper panel) or E-box from dhfr (lower panel) and the indicated competitor oligonucleotides. Arrow indicates the shift of c-Myc/Max oligonucleotide complexes. Data represent at least three independent assays. *p < 0.05, **p < 0.001.

study. Here, we addressed the role of c-Myc in B cell differentiation in vivo and found that c-Myc regulates this process in part by conferring identity to early B lymphocyte precursors.

The generation of mb1-fl/fl and mx-fl/fl mouse models allowed us to define the requirements for c-Myc in B lymphocytes at distinct developmental stages. Conditional inactivation of c-myc in mb1-fl/fl mice showed that this gene is required at least from pre-B to immature B cell stages; we observed a reduction in pre-B cells and increased apoptosis in these cells (Figs. 1, 2). Fewer pro-B cells are affected than pre-B cells, probably due to the time at which c-myc deletion occurs in these mice. The reduced number of cells that express GFP in gfp-mb1-fl/fl mouse BM probably reflects increased apoptosis in c-Myc−/− B lymphocytes as well as the accessibility of both loci to Cre recombinase (47). A similar block during transition from pro-B to pre-B cell has been described for the mb1-cre-mediated deletion of c-myb, a known gene regulating c-myc (48). Our results are in agreement and provide evidence of a more prominent role of c-Myc in collaboration with C-Myc in the regulation of these processes via EBF-1.

The increased apoptosis observed at all of the developmental stages except in mature B cells (21) and the inability of mx-fl/fl (34) and mb1-fl/fl mice to generate B220+IgM+ cells in vivo and in vitro (Fig. 2, Supplemental Fig. 3) show the need for c-Myc in B cell generation and maintenance during differentiation. The c-Myc requirement in hematopoietic stem cell differentiation (34, 49) probably contributes to the decreased number of early B cell precursors in mx-fl/fl mouse BM.

The role of c-Myc in regulating the G1-S transition of the cell cycle in different cell types has been widely studied (19). At early stages, we observed that B cells lacking c-Myc retain limited proliferative capacity in both mb1-fl/fl and mx-fl/fl mice (Fig. 3A, 3B). This ability to proliferate in the absence of c-Myc has been reported for other cell types (28, 49, 50). It is possible that cells are already cycling and that c-myc is deleted at stages when the protein is less critical to continue through the cell cycle. This might be more relevant at the transition between pro- and pre-B cell stages, when extensive expansion occurs (Fig. 3A, 3B). Alternatively, this could reflect distinct c-Myc requirements for cell proliferation, depending on the developmental stage.

We did not observe c-Myc−/− dependent transcriptional regulation of tcfe2a or pax-5 promoters in luciferase reporter assays (Supplemental Fig. 6). The reduced tcfe2a gene expression in c-Myc−/− deficient B lymphocytes nonetheless suggests an indirect effect of c-Myc on tcfe2a regulation. It remains to be determined whether E2A expression in c-Myc−/− deficient B lymphocytes is sufficient to promote B lymphocyte differentiation. We identified ebf-1 as a previously unreported c-Myc target gene. The contribution of the ebf-1 α promoter to the total level of ebf-1 transcripts is small compared with that of the β promoter. However, we believe that this contribution is essential at early stages of B cell differentiation due to the complex regulation of ebf-1 expression as described previously (44). The activity of the ebf-1 α promoter will induce expression of pax-5, which in turn will activate the ebf-1 β promoter. In c-Myc−/− deficient B cells, activation of the ebf-1 α promoter will be compromised, and therefore the total amounts of EBF-1 mRNA will be reduced dramatically.

Our results indicate that c-Myc regulates cell proliferation and survival in developing B lymphocytes; c-Myc function thus is not restricted to the regulation of ebf-1 expression in B cell differentiation.
FIGURE 6. Ectopic expression of EBF-1 rescues B lymphocyte differentiation in c-Myc–deficient B lymphocytes. A, Lin<sup>−</sup> cells from mb1-fl/fl and mb1-fl/+ mouse BM were isolated and infected with ebf-1-gfp-expressing or gfp retrovirus. Cells were harvested after 6 d, Ab-stained, and analyzed by flow cytometry. Data represent at least three independent assays. B, V(D)J recombination in c-Myc–deficient B lymphocytes ectopically expressing EBF-1. Genomic PCR was performed on DNA from sorted B220<sup>+</sup>CD19<sup>+</sup>GFP<sup>+</sup> or B220<sup>+</sup>CD19<sup>+</sup>GFP<sup>+</sup> cells infected with EBF-1 or GFP control retrovirus from a pool of four mb1-fl/fl and three mb1-fl/+ control mice. C, Cell cycle analysis of sorted B220<sup>+</sup>CD19<sup>+</sup>GFP<sup>+</sup> cells infected with EBF-1–expressing retrovirus as in A. Cells were sorted and stained with propidium iodide. Data represent two independent experiments. D, Gene expression of pax-5, ebf-1, tcf2a, and n-myc in ebf-1–infected cells. Cells were infected as in A, sorted, and analyzed by qPCR. A pool of three mice of each genotype was used.
The observation that c-Myc–deficient pre-B cells undergo a slight reduction but normal V(D)J recombination despite decreased tcfe2a (4, 5), ebf-1 (6), and pax-5 (10, 51) expression probably reflects cell pool heterogeneity while undergoing c-myc deletion. This became more evident when we compared c-Myc–deficient and pax-5<sup>2</sup>/2 B cells. Despite reduced pax-5 expression in c-Myc–deficient B lymphocytes, we observed minimal differences in the levels of V<sub>H</sub>proximal- and V<sub>H</sub>distal-to-DHJH recombination (10). In c-Myc–deficient B cells, enforced expression of EBF-1 induces surface expression of CD19 and slightly increases the levels of D to J recombination in these cells (Fig. 6B). These results might reflect a broad rather than a specific effect of c-Myc deficiency on V(D)J machinery (51).

Unlike pax-5<sup>−/−</sup> B cells (52), c-Myc–deficient B lymphocytes were unable to differentiate to other cell lineages in vivo and in vitro (Supplemental Fig. 5; data not shown). Although we did not detect B220<sup>+</sup> GFP<sup>+</sup> cells in the thymus, BM, or spleen of gfp-<i>mb1-fl/fl</i> mice (data not shown), it is nonetheless possible that lack of c-Myc confers on B lymphocytes the ability to differentiate to other cell lineages. To test this, the increased viability of c-Myc–deficient B cells is essential. Our attempts to rescue c-Myc–deficient B lymphocytes from apoptosis by breeding mx<sup>-fl/fl</sup> with E<sub>m</sub>-bcl-2 transgenic mice (53) were unsuccessful (data not shown).

The capacity of EBF-1 to induce pax-5 gene expression (41, 54, 55) and to activate the B cell transcription program could explain its ability to promote B cell differentiation in c-Myc–deficient cells in vitro, despite the large number of genes regulated by c-Myc (Fig. 6). Pax-5 expression in ik-mb1-fl/fl mice nonetheless did not rescue B cell differentiation (Supplemental Fig. 6). This might be attributed to the brief time frame available for the expression of normal Pax-5 levels before cell death after c-Myc deletion (Supplemental Fig. 7). In contrast, Pax-5 expression in ik-mx-fl/fl mice contributed to a significant increase in the number of c-Myc–deficient immature B cells and to cell surface expression of the Pax-5 target CD19 (Fig. 7A). The pro- and pre-B cell numbers did not increase significantly in ik-mx-fl/fl mice as observed by Souabni et al. (30) (Fig. 7B). In our experimental model, developing B lymphocytes show increased cell death upon deletion of c-myc (Fig. 2). This effect probably makes it more difficult to increase

![FIGURE 7. Pax-5 expression contributes to differentiation of c-Myc–deficient B lymphocytes. A, Flow cytometry analysis of ik-mx-fl/fl and control mouse BM. B, Absolute numbers of B cell subpopulations in BM of mice in A. ik-mx-fl/fl, n = 5; fl/fl, n = 6; mx-fl/fl, n = 4. C, c-myc deletion in sorted populations from mice of the indicated genotypes. Data represent two independent experiments. **<i>p < 0.01</i>.](http://www.jimmunol.org/)
B cell numbers in our system than in that of Souabni et al. (30), where Pax-5 is overexpressed in a normal background. Our system does not allow us to control when c-myc deletion and/or pax-5 expression occur with respect to each other. Moreover, c-Myc affects B lymphocytes depending on the differentiation stage (21, 22). Altogether, these effects might account for the differences in cell number in immature B lymphocytes in ik-mx/fl/fl mice.

Our results identified ebf-1 as an unreported c-Myc target gene and illustrate a novel c-Myc function in the regulation of B lymphocyte differentiation. Through ebf-1 activation, c-Myc regulates differentiation by promoting B cell identity. These data show that c-Myc not only regulates ebf-1 but also affects multiple biological functions during B lymphocyte differentiation such as cell survival or proliferation. The capacity of c-Myc to regulate these functions has been widely studied (16, 19).

Finally, this study places c-Myc within the context of transcription factors essential for B lymphocyte differentiation by linking this transcription factor to the EBF-1/Pax-5 pathway. On the basis of these data, a model emerges for transcriptional regulation of B lymphocyte differentiation in which c-Myc acts by regulating B or T cell-specific transcription factors. This model postulates a requirement for one or more additional factor(s) to allow c-Myc to discriminate between B and T cell lineages.

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Disclosures
The authors have no financial conflicts of interest.

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