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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 this 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 ($B220^+c\text{-}Kit^+$) 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 *tcfe2a* (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_{distal}-to-D_HJ_H rearrangement (10). Pax-5 regulates the expression of the B cell-specific genes *cd19* (11), *blnk* (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 G_0/G_1-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

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Abbreviations used in this article: as, antisense; BM, bone marrow; ChIP, chromatin immunoprecipitation; pIpC, polyinosinic-polycytidylic acid; qPCR, quantitative PCR; s, sense.

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$^{fl/fl}$;mx-cre^{+}* mice was described (28). To generate *c-myc$^{fl/fl}$;mb1$^{cre/+}$* mice, *c-myc$^{fl/fl}$* mice were bred with *mb1$^{cre/+}$*mice (29), and progeny were crossbred to yield homozygous (*c-myc$^{fl/fl}$;mb1$^{cre/+}$*) and control mice (*c-myc$^{fl/+}$;mb1$^{cre/+}$* or *c-myc$^{fl/fl}$;mb1$^{+/+}$*). *c-myc$^{fl/fl}$;mx-cre^{+}* or *c-myc$^{fl/fl}$;mb1$^{cre/+}$* mice were bred with *ik$^{neo/+}$* mice (30) to generate *ik$^{neo/+}$;c-myc$^{fl/fl}$;mx-cre^{+}* or *ik$^{neo/+}$;c-myc$^{fl/fl}$;mb1$^{cre/+}$* mice, respectively. Progeny were crossed to generate homozygous (*ik$^{neo/+}$;c-myc$^{fl/fl}$;mx-cre^{+} or ik$^{neo/+}$;c-myc$^{fl/fl}$;mb1$^{cre/+}$*) and control mice (*ik$^{neo/+}$;c-myc$^{fl/fl}$;mx-cre^{-} or ik$^{neo/+}$;c-myc$^{fl/+}$;mb1$^{cre/+}$*). Mice were genotyped using a PCR-based analysis of tail genomic DNA (28). Primers hcre-DIR (5′-ACC TCT GAT GAA GTC AGG AAG AAC-3′), hcre-REV (5′-GGA GAT GTC CTT CAC TCT GAT TCT-3′), mb1in1 (5′-CTG CGG GTA GAA GGG GGT C-3′), and mb1in2 (5′-CCT TGC GAG GTC AGG GAG CC-3′) were used to amplify *mb1-cre* (hcre-DIR and hcre-REV) and *mb1-wt* alleles (mb1in1 and mb1in2). The knock-in allele (*ik$^{neo/+}$*) was identified as described (30). *c-myc$^{fl/fl}$;mx-cre^{+}* or *c-myc$^{fl/fl}$;mb1$^{cre/+}$* mice were bred with *rosa26$^{gfp/gfp}$* "reporter" mice (31) to generate *c-myc$^{fl/fl}$;mx-cre^{+};rosa26$^{gfp/gfp}$* or *c-myc$^{fl/fl}$;mb1$^{cre/+}$;rosa26$^{gfp/gfp}$* mice, respectively. The *rosa26^{gfp}* allele was genotyped as described (31).

Polyinosinic-polycytidylic acid injections

To induce *c-myc* deletion in *c-myc$^{fl/fl}$;mx-cre^{+}* and *ik$^{neo/+}$;c-myc$^{fl/fl}$;mx-cre^{+}* mice, 4- to 6-wk-old animals received three i.p. injections of polyinosinic-polycytidylic acid (pIpC; 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^{-}DX5^{-}B220^{+}c-Kit^{+}IgM^{-}) and pre-B cells (Ly-6c^{-}NK1.1^{-}DX5^{-}B220^{+}CD25^{+}IgM^{-}). 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 Biotechnology Associates) were conjugated with either PE or biotin. Allophycocyanin-anti–CD19 Ab was from Becton Dickinson. PE-anti–CD25, biotin-anti–CD43, PE-anti–CD117, and biotin-anti–pre-BCR Abs were all from BD Pharmingen. Allophycocyanin–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 dump 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^{+}c-Kit^{+}IgM^{-}) and pre-B cells (Ly-6c^{-}NK1.1^{-}DX5^{-}B220^{+}CD25^{+}IgM^{-}) from *mb1-fl/fl* and *mb1-fl/+* 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 *mx-fl/fl* and *fl/fl* control mice.

Retrovirus production and transduction

Plat-E cells were seeded (2 × 10^{6} cells) in 6-cm plates 18–24 h before transfection with MIG-RI or MIG-EBF 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 low-protein-binding syringe filter (Pall Life Sciences). Lin^{-} precursors were purified from total BM suspensions with streptavidin Dynabeads (Invitrogen) by incubation with a mixture of biotinylated Abs to lineage markers (B220, IgM, CD4, CD8, Ter119, Gr1, and CD11b; all from BD Pharmingen). Cells were cultured at a concentration of 10^{6} cells per milliliter in 24-well plates in DMEM containing 10% heat-inactivated FBS and 1 mM L-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 resuspended in 1 ml fresh retroviral supernatant, supplemented with 10 μg/ml polybrene (Sigma-Aldrich) and cytokines as above. Cells were centrifuged (1136 × *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 Primatone RL (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 V_H-specific primers from Fuxa et al. (32); DJ primers were from Ehlich 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 V_HJ558-J_H3 band was cloned in PCRII-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 (s) (5′-AGTACGGGAATGTGCTCTCC-3′) and antisense (as) (5′-GGACTTGAATGCGTGGATTT-3′) for *cd19*, E2A s (5′-ATACAGCGAAGGTGCCCACT-3′) and E2A as (5′-CTCAAGGTGCCAACACTGGT-3′) for *tcfe2a*, EBF-1 s (5′-CTATGTGCGCCTCATCGACT-3′) and EBF-1 as (5′-CATGATCTCGTGTGTGAGCAA-3′) for *ebf-1*, Flt3 s (5′-CAGCCGCACTTTGATTTACA-3′) and Flt3 as (5′-GGCTTCGCTCTGAATATGGA-3′) for *flt3*, Ikaros s (5′-TTGTGGCCGGAGCTATAAAC-3′) and Ikaros as (5′-TGCCATCTCGTTGTGGTTAG-3′) for *ikaros*, Lef-1 s (5′-TCAGTCATCCCGAAGAGGAG-3′) and Lef-1 as (5′-CTCTGGCCTTGTCGTGGTAG-3′) for *lef-1*, Pu-1 s (5′-GGGATCTGACCAACTGGAG-3′) and Pu-1 as (5′-GCTGCCCACGAAGGAGTAGTA-3′) for *spfi1*, and Rag1 s (5′-GTTGCTATCTCTGTGGCATCG-3′) and Rag1 as (5′-AATTTCATCGGGTGCAGAAC-3′) for *rag1*. For the remainder of the genes, predesigned Applied Biosystems Micro Fluidic cards were used. Each gene was analyzed in triplicate. cDNA samples and reagents were run on an Applied Biosystems Prism 7900HT. Data were analyzed with SDS2.2 sequence detection systems.

Luciferase activity assay

The murine *ebf-1* α promoter was amplified by PCR using the primers Ebf-1α5 (5′-TAAGAGCGCGGAACTGTCC-3′) and Ebf-1α3 (5′-GCTGAAGAATCTGCCAGAAGTT-3′), cloned into the EcoRI site of the TOPO 2.1 vector (Invitrogen), and subcloned into the NheI/BglII site of the pGL3-Control vector (Promega) upstream of the luciferase gene to generate the vector pGL3-Ebf-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-Ebf-1α or pGL3-Control vector and increasing amounts of pRV-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). L1-2 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 × 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 $2^{(Ct\ input\_Ct\ Ab)}$. Primers flanking E-box5 were: EB5-FW (5′-CCTCAGCTCGTTCTGAGAGG-3′) and EB5-REV(5′-ACTCGCAGGAGGTAGAGAACG-3′).

EMSA

Assays were performed with labeled double-stranded oligonucleotides encompassing E-boxes from the *dhfr* and *ebf-1* α promoters. pcDNA3-c-

Myc 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-764x) or 1 μg anti–c-MYB (sc-517x) Abs (both from Santa Cruz Biotechnology) were used. Double-stranded oligonucleotides used were: DHFR-I-wt (5′-GGCGCGACACCCACGTGCCCT-3′ and 5′-AGAGAGGGCACGTGGGTGTCG-3′), EB5-wt (5′-GGTCCTACCCACGTTGACTGCAGT-3′ and 5′GAGACTGCAGTCAACGTGGGTAGGA-3′), EB5-mut (5′-GGTCCTACCCTTGCTGACTGCAGT-3′ and 5′-GAGACTGCAGTCAGCAAGGGTAGGA-3′), EB505-wt (5′-CGTTTCCTCACCTGTACAATGGGAGTGG-3′ and 5′-GTCCACTCCCATTGTACAGGTGAGGAAA-3′), EB505-mut (5′-CGTTTCCTCTCTTATACAATGGGAGTGG-3′ and 5′-GTCCACTCCCATTGTATAAGAGAGGAAA-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 *mb-1*$^{cre/+}$ knock-in (29), *mx-cre* transgenic (36), and *rosa26*$^{gfp/gfp}$ 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* mice and in *gfp-mx-fl/fl* mice, the *rosa26*$^{gfp/gfp}$ allele expresses GFP after activation of Cre recombinase (31) (Supplemental Fig. 1*A*).

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. 1*A*, 1*B*, Supplemental Fig. 1*B*). Analysis of Hardy fractions in these mice also revealed a developmental defect and a decrease in absolute numbers in Fractions B, C, and C′ (large pre-B cells), which is consistent with the time in which deletion of *c-myc* occurs (Fig. 1*C*, 1*D*). 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. 1*E*). *mb1-fl/fl* spleens showed a 34-fold decrease (0.2×10^6 versus 8.1×10^6) in the number of mature B lymphocytes ($B220^+IgM^+$) compared with those of controls (Fig. 1*E*). Genomic PCR analysis of these cells confirmed *c-myc* deletion (Supplemental Fig. 1*D*, 1*E*). 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. 2*A*). 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. Analysis of Hardy fractions showed a decrease in the absolute numbers from fractions A to E (Fig. 1*F*, 1*G*, Supplemental Fig. 2*E–G*). Fraction F seems to be less affected, which is consistent with our previous results in mature B cells (21) (Fig. 1*F*, 1*G*). Annexin V staining showed an increase (35.8 versus 14.1%) in the relative numbers of apoptotic pro- and pre-B ($B220^+IgM^-$) and immature B cells ($B220^+IgM^+$) (77.9 versus 27.6%) in *mx-fl/fl* mouse BM compared with those in controls (Fig. 2*B*). Recirculating mature ($B220^{++}IgM^+$) B lymphocytes in *mx-fl/fl* mouse BM survived in the absence of c-Myc (26.6 versus 31.5%), which is consistent with results for *c-myc*$^{fl/fl}$*;cd19*$^{cre/+}$ mice (21), indicating that *c-myc* is dispensable for mature B lymphocyte maintenance (Figs. 1*F*, 2*B*). The lack of early hematopoietic precursors (28) could also contribute to the decrease in the number of recirculating mature B cells in *mx-fl/fl* mouse BM (data not shown). These results indicate that c-Myc is necessary for the generation of pro- and pre-B cells and for maintenance of immature B lymphocytes.

Cell proliferation in developing c-Myc–deficient B lymphocytes

c-Myc promotes proliferation in many cell types, including B lymphocytes, by regulating cell cycle genes (19). The pro- to pre-B cell transition also is characterized by cell expansion (39). 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. 3*A*). The *mx-fl/fl* mouse pro- and pre-B cells showed similar proliferative capacities (Fig. 3*B*). We did not observe significant differences in proliferation in immature lymphocytes from *mx-fl/fl* mice (Fig. 3*B*). We concluded that developing B lymphocytes have a reduced capacity to proliferate in the absence of c-Myc in vivo.

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

Sequential rearrangement of the V(D)J 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(D)J rearrangement by genomic PCR in sorted pro- and pre-B cells. We did not observe significant differences in D_H-to-J_H and $V_{Hproximal}$- and $V_{Hdistal}$-to-D_HJ_H rearrangements in purified pro-B cells from *mb1-fl/fl* mouse BM compared with those of control cells (Fig. 3*C*, *upper panel*, and Fig. 3*D*, $p > 0.05$). In contrast, purified pre-B cells from mutant mice showed a slight decrease in $V_{Hproximal}$- (1.9-fold) and $V_{Hdistal}$-to-D_HJ_H (2.4-fold) rearrangements compared with those of control mice (Fig. 3*C*, *lower panel*, and Fig. 3*D*). 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(D)J rearrangements from *mb1-fl/fl* and *mb1-fl/+* mouse B 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 *tcfe2a*, *ebf-1*, *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. 4*A*). Inter-

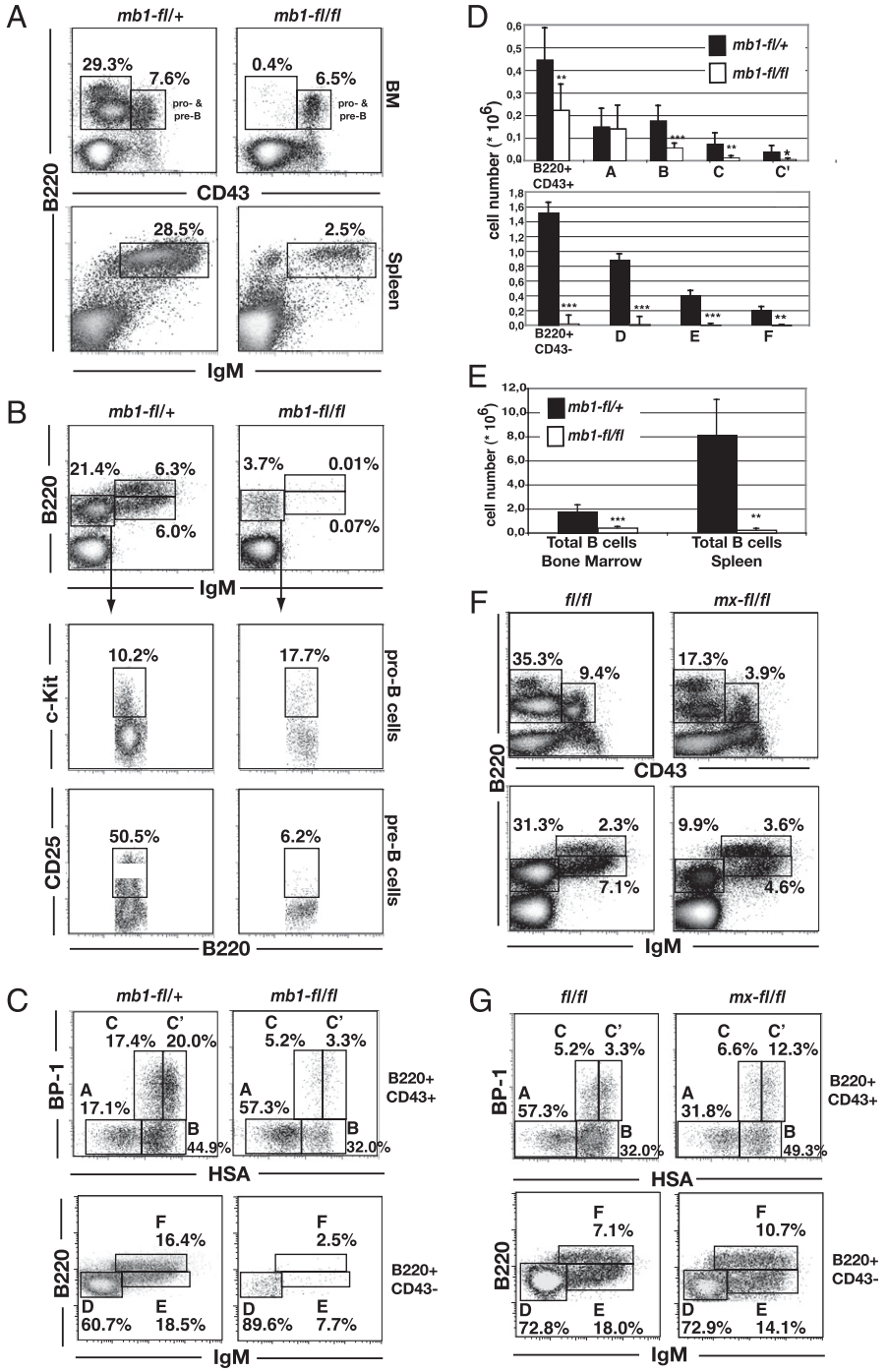


FIGURE 1. c-Myc is necessary for B lymphocyte differentiation. *A* and *B*, B lymphocyte differentiation is blocked at the pre-B cell stage in *mb1-fl/fl* mouse BM. Single-cell suspensions were prepared from *mb1-fl/fl* and *mb1-fl/+* mice, stained, and analyzed by flow cytometry (see *Materials and Methods*). Cells were defined as pro-B (Ly-6c$^-$NK1.1$^-$DX5$^-$B220$^+$c-Kit$^+$IgM$^-$) and pre-B cells (Ly-6c$^-$NK1.1$^-$DX5$^-$B220$^+$CD25$^+$IgM$^-$). *C*, Flow cytometry analysis of Hardy fractions in BM of *mb1-fl/fl* and *mb1-fl/+* control mice. B220$^+$CD43$^+$ and B220$^+$CD43$^-$ gates and NK and dendritic cell discrimination were as in *A*. *D* and *E*, Absolute numbers of B lymphocytes in *mb1-fl/fl* and control mouse BM (n = 7) and spleen (n = 3). *F*, Flow cytometry analysis of B lymphocytes from pIpC-injected *mx-fl/fl* and *fl/fl* mouse BM. *G*, Flow cytometry analysis of Hardy fractions in BM of *mx-fl/fl* mice. Data represent one of three or more independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

estingly, *tcfe2a*$^{-/-}$, *ebf-1*$^{-/-}$, and *pax-5*$^{-/-}$ 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. 4*A*). 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. 4*B*, 4*C*). 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*$^{-/+}$ 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. 4*C*).

Consistent with these results, gene expression of the Pax-5 target genes *blnk* (12), *cd79a*, *cd79b*, and *n-myc* (41) was reduced in B220$^+$IgM$^-$ cells (pro- and pre-B cells) from *mb1-fl/fl* mice (Supplemental Fig. 4*A*). Similarly, pro- and pre-B cells from *gfp-mx-fl/fl* mice showed reduced *cd79b*, *cd79a*, and *pax-5* expression (Supplemental Fig. 4*B*). To rule out contamination with non-B cells, we tested *c-myc* and *flt3* expression in pro- and pre-B cells purified using a mixture of Abs to pro-B (Ly-6c$^-$NK1.1$^-$DX5$^-$B220$^+$c-Kit$^+$IgM$^-$) and pre-B cells (Ly-6c$^-$NK1.1$^-$DX5$^-$B220$^+$

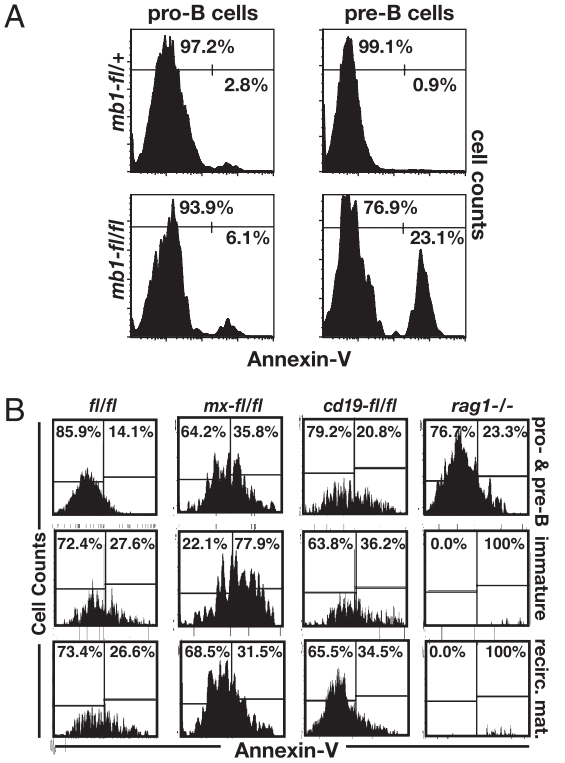
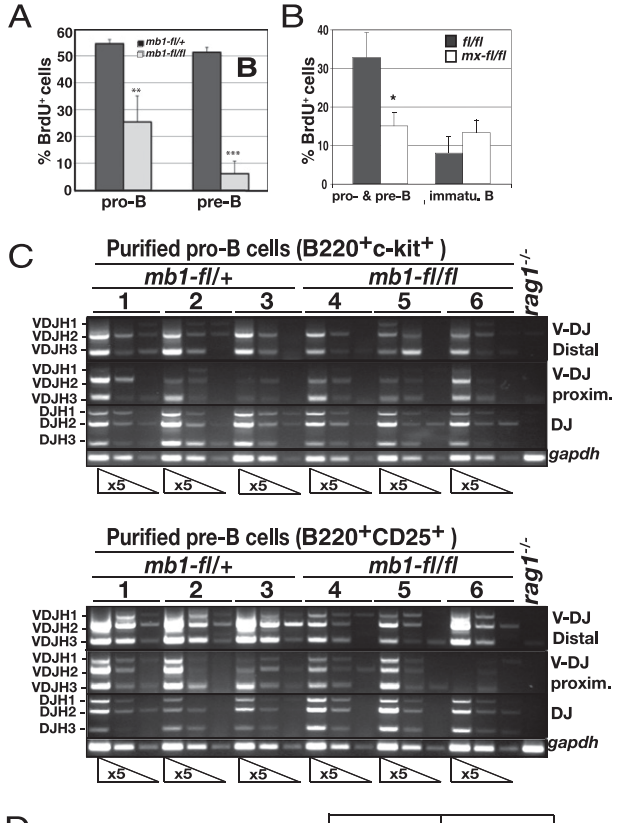


FIGURE 2. Increased apoptosis in developing c-Myc–deficient B lymphocytes. *A*, Apoptosis in pro- and pre-B cells in *mb1-fl/fl* mice. Cells were Ab- and annexin V-stained and gated as in Fig. 1*B*. *B*, Pro-, pre-, and immature B lymphocytes die by apoptosis after *c-myc* deletion in *mx-fl/fl* mice. BM cells were stained as in Fig. 1*F*, and B cell populations were analyzed by flow cytometry using gates as in Fig. 1 *F*. Data represent three independent experiments.



D

		IntDen Fold	*p-value*
pro-B	Distal V-DJ	-1.27	0.107 (NS)
	Prox.V-DJ	1.04	0.831 (NS)
	D-J	-1.21	0.183 (NS)
pre-B	Distal V-DJ	-2.36	1.22E-05 (***)
	Prox.V-DJ	-1.86	0.010 (*)
	D-J	1.22	0.453 (NS)

FIGURE 3. Cell proliferation and V(D)J recombination of c-Myc–deficient B lymphocytes. *A*, Decreased proliferation of sorted pro- and pre-B cells from *mb1-fl/fl* mice. Cells were stained as in Fig. 1*B*, and BrdU incorporation was measured by flow cytometry in pro-B (Ly-6c$^-$NK1.1$^-$DX5$^-$B220$^+$c-Kit$^+$IgM$^-$) and pre-B cells (Ly-6c$^-$NK1.1$^-$DX5$^-$B220$^+$CD25$^+$IgM$^-$) in the BM. *B*, Pro- and pre-B cells from *mx-fl/fl* mouse BM. Mice were injected with BrdU 2 h before analysis. $n = 3$ for *mb1-fl/fl* and $n = 5$ for *mx-fl/fl*. *C*, V(D)J recombination analysis in sorted pro- and pre-B cells from *mb1-fl/fl* or control mice. Genomic PCR from sorted BM pro- and pre-B cells defined as in Fig. 1*B* was performed using specific primers to detect D-to-J_H or V_H-to-DJ_H rearrangements (see *Materials and Methods*). Three mice of each genotype were analyzed; DNA from *rag-1*$^{-/-}$ mice was included as a negative control. *D*, VDJ rearrangement levels were quantified by measuring the fluorescence intensity of the amplified PCR fragments in the agarose gel using ImageJ software. Fold IntDen indicates the integrated intensity average ratio of *mb1-fl/fl* versus that of control samples, including the three 5-fold dilutions of three different mice from each genotype, after GAPDH normalization. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

CD25$^+$IgM$^-$) (Supplemental Fig. 4*C*, 4*D*). 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* β promoter did not activate the *luciferase* gene in a c-Myc dose-dependent manner (Supplemental Fig. 5*A*, 5*B*). 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 (2.5-fold) and in the L1-2 B cell line (2-fold) (Fig. 5*A–C*). Luciferase assays showed that site-directed mutagenesis of the E-box5 (EBΔ5), located 200 bp upstream of the transcription start site, completely abolished basal promoter activity and c-Myc-dependent *ebf-1* transactivation in both cell lines (Fig. 5*A–C*). c-Myc-dependent transactivation was not observed with genomic regions containing the *pax-5* or *tcfe2a* promoters (Supplemental Fig. 5*C*, 5*D*).

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. 5*D*). 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. 5*E*, *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. 5*E*, *lower panel*). Altogether, these data show that c-

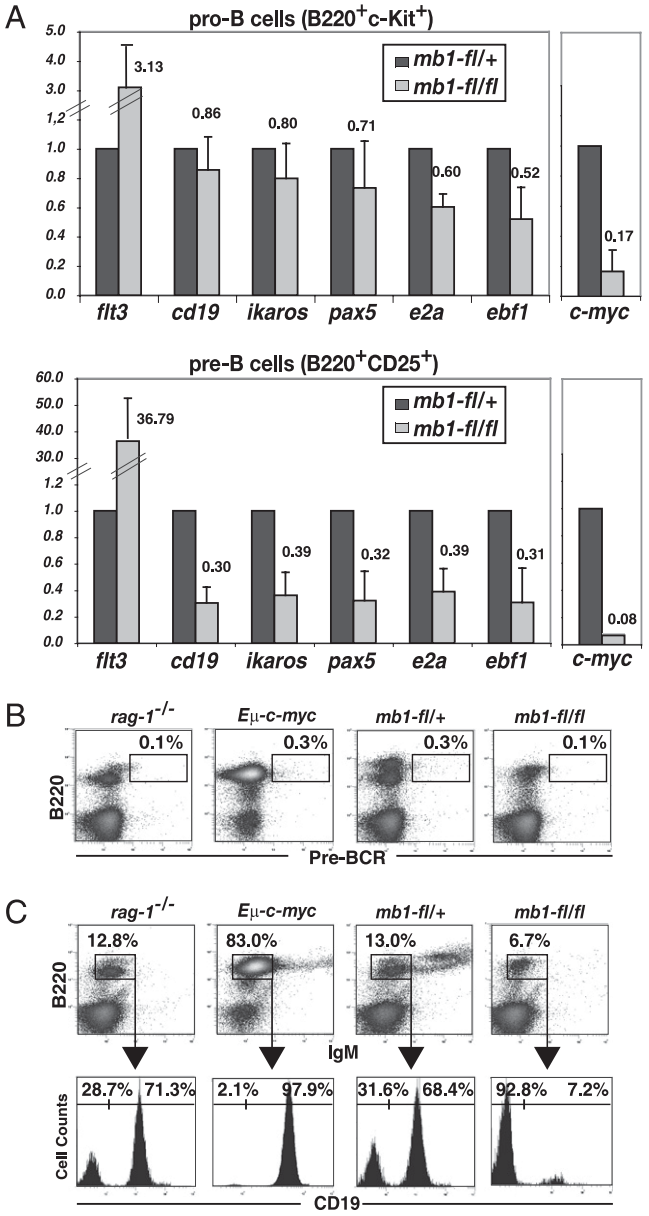


FIGURE 4. Gene expression in c-Myc–deficient B lymphocytes. *A*, qPCR of sorted pro- and pre-B cells from *mb1-fl/fl* and *mb1-fl/+* control mouse BM. Each panel shows an independent experiment (mean ± SD for three mutant and three control mice); numbers indicate the *x*-fold change ($2^{-\Delta Ct}$). *B* and *C*, Flow cytometry analysis of pre-BCR and CD19 surface expression on *mb1-fl/fl* and *mb1-fl/+* control mouse BM B lymphocytes. *c-myc* transgenic (*Eμ-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. 6*A*). 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. 6*A*). We observed an increase in D_H-to-J_H rearrangements in EBF-1–infected $B220^+CD19^+GFP^+$cells compared with those in $B220^+CD19^-GFP^+$ cells infected with control retrovirus (Fig. 6*B*). V_H-to-D_HJ_H rearrangements were hardly detected in both populations infected with either retrovirus (Fig. 6*B*). 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. 6*C*).

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 *tcfe2a* (Fig. 6*D*). 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 $ik^{neo/+}$ mice (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, pIpC 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-mb1-fl/fl* mice were unsuccessful probably due to the low levels of *pax-5* expression in these mice (Supplemental Figs. 7*A–C*, 8). Flow cytometry analysis of B cell populations in the BM of pIpC-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. 7*A*). 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

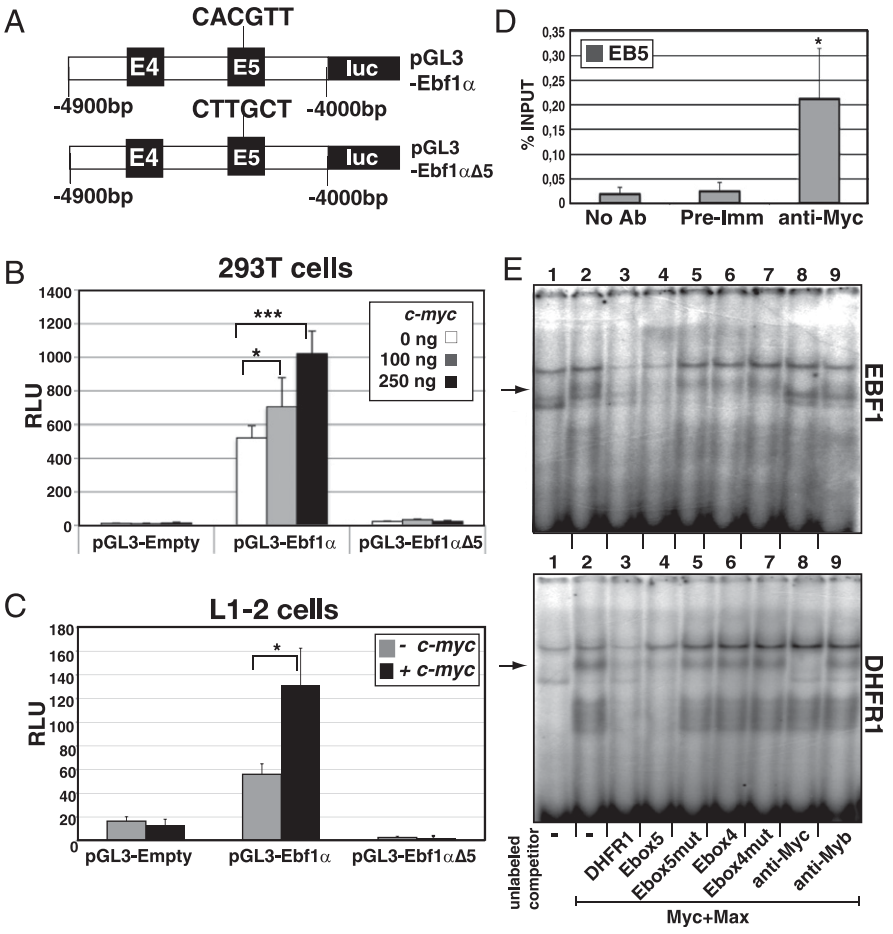


FIGURE 5. c-Myc transcriptionally regulates *ebf-1*. *A*, Luciferase reporter constructs. *B* and *C*, c-Myc dose-dependent activation of *ebf-1* in HEK 293T and L1-2 cells (B cells) in transient transfection assays. Luciferase activity was normalized with *Renilla* activity (relative luciferase units, 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. *Lane 1*, Negative control without c-Myc and Max. *Lane 2*, No competitor. *Lane 9*, Negative control with anti–c-Myb Ab. 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–deficient 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-Myb 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 G_1-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. 3*A*, 3*B*). 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. 3*A*, 3*B*). 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.

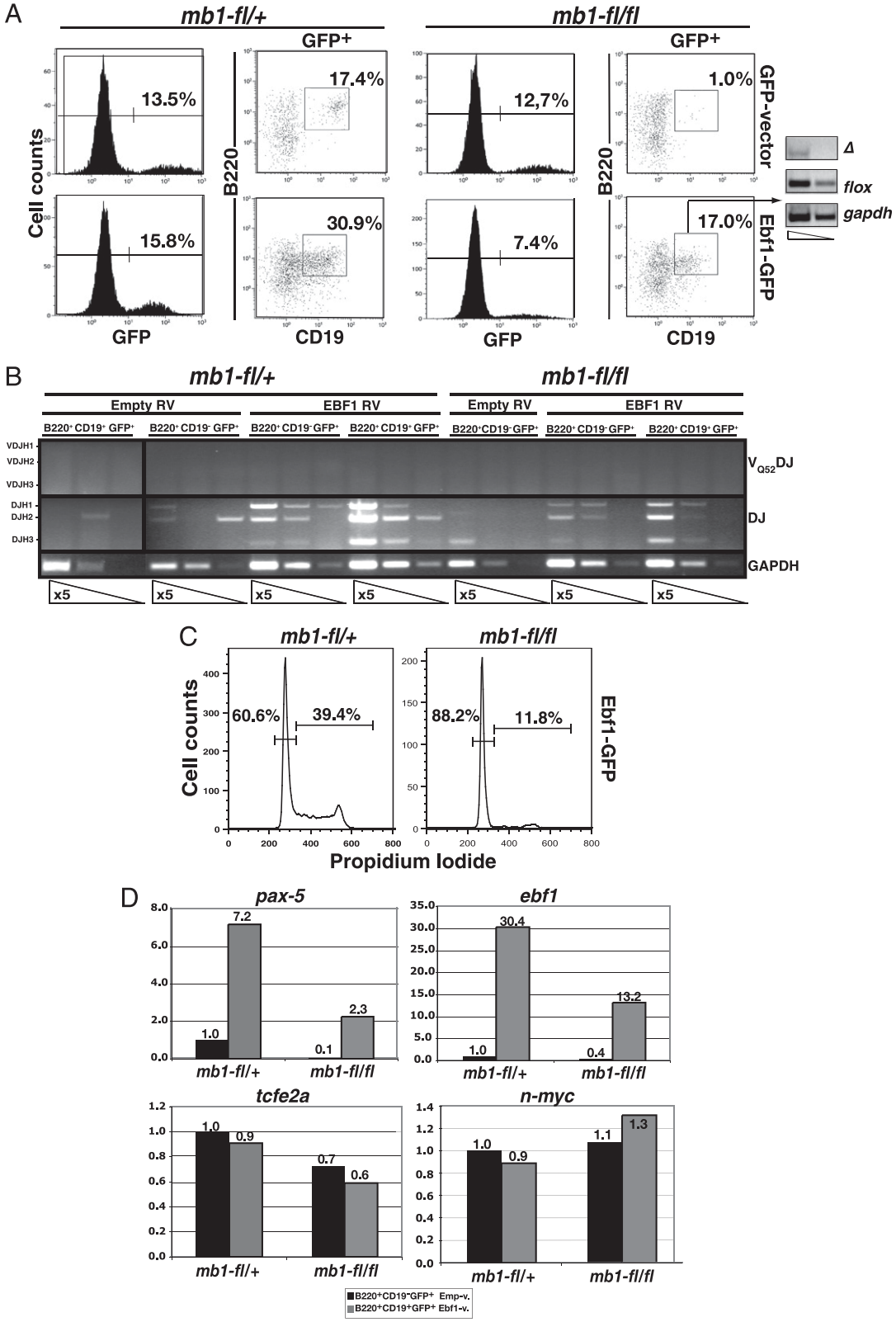


FIGURE 6. Ectopic expression of EBF-1 rescues B lymphocyte differentiation in c-Myc–deficient B lymphocytes. *A*, Lin$^-$ cells from *mb1-fl/fl* and *mb1-fl/+* mouse BM were isolated and infected with *ebf-1-gfp*-expressing or *gfp* retrovirus. Cells were harvested after 6 d, Ab-stained, and analyzed 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$^+$CD19$^+$GFP$^+$ or B220$^+$CD19$^-$GFP$^+$ 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$^+$CD19$^+$GFP$^+$ 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*, *tcfe2a*, 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.

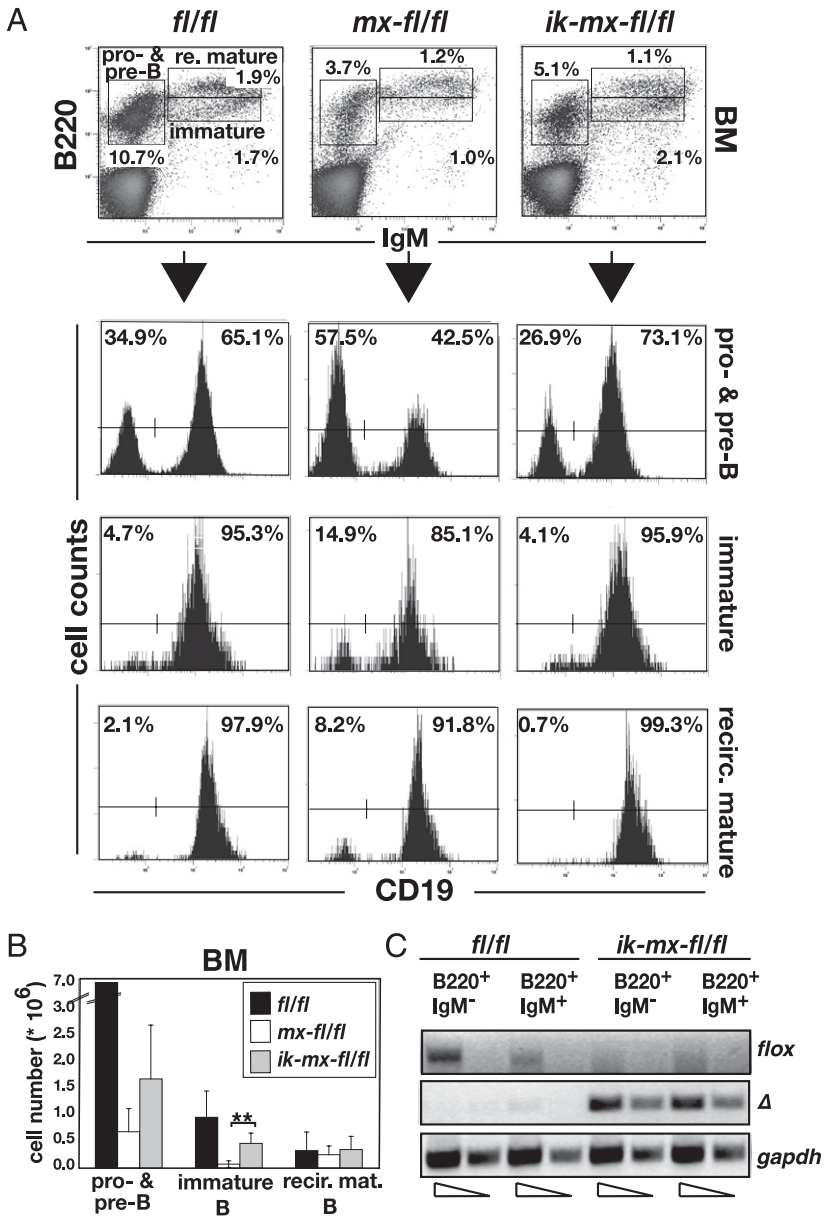


FIGURE 7. Pax-5 expression contributes to differentiation of c-Myc–deficient B lymphocytes. *A*, Flow cytometry analysis of *ik-mx-fl/fl* and control mouse BM. *B*, Absolute numbers of B cell subpopulations in BM of mice in *A*. *ik-mx-fl/fl*, $n = 5$; *fl/fl*, $n = 6$; *mx-fl/fl*, $n = 4$. *C*, *c-myc* deletion in sorted populations from mice of the indicated genotypes. Data represent two independent experiments. $^{**}p < 0.01$.

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*$^{-/-}$ B cells. Despite reduced *pax-5* expression in c-Myc–deficient B lymphocytes, we observed minimal differences in the levels of $V_{Hproximal}$- and $V_{Hdistal}$-to-D_HJ_H 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. 6*B*). These results might reflect a broad rather than a specific effect of c-Myc deficiency on V(D)J machinery (51).

Unlike *pax-5*$^{-/-}$ 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^{-}GFP^{+} cells in the thymus, BM, or spleen of *gfp-mb1-fl/fl* 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-fl/fl* with Eμ-*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. 7*A*). 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. 7*B*). 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

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 us 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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