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c-Myb Is Required for Pro-B Cell Differentiation

Shawn P. Fahl,* Rowena B. Crittenden,* David Allman,‡ and Timothy P. Bender**

The c-Myb transcription factor is required for normal adult hematopoiesis. However, the embryonic lethality of Myb-null mutations has been an impediment to identifying roles for c-Myb during lymphocyte development. We have used tissue-specific inactivation of the Myb locus in early progenitor cells to demonstrate that c-Myb is absolutely required for the differentiation of CD19⁺ B-lineage cells and that the expression of cell surface markers and the ordered rearrangement of Ig H and L chain gene segments (1). Hematopoietic stem cells give rise to progenitor B cells (pro-B cells) that undergo rearrangement of the Ig H chain locus. Successful H chain rearrangement leads to expression of μH chain, which associates with surrogate L chain and the signaling molecules Ig-α and Ig-β to form the pre-BCR complex. Signaling through the pre-BCR results in clonal expansion of cytoplasmic μ⁺ cells and differentiation to the small pre-B cell stage of differentiation. Small pre-B cells undergo rearrangement of the Ig L chain loci. Pre-B cells that produce L chain, paired with the μ H chain, express membrane-bound IgM and are referred to as immature B cells.

The pathway from hematopoietic stem cells (HSCs) to the committed B cell progenitor involves the sequential generation of multipotent progenitor cells (MPPs) that can give rise to each hematopoietic lineage and lymphoid-multipotent multipotent progenitors (LMPPs) (2). LMPPs can give rise to lymphocytes, granulocytes, and macrophages but have generally lost the ability to generate erythrocytes and megakaryocytes (3, 4). HSCs, MPPs, and LMPPs are distinguished by expression of Flt3 (3). LMPPs serve as precursors for common lymphoid progenitors (CLPs), which express the IL-7R and retain broad differentiation potential for the lymphocytic lineage but not granulocytes or macrophages (5, 6). CLPs give rise to a population of cells referred to as pre-pro-B cells, fraction A, or CLP2 cells that express B220 on the surface and represent the first clear B-lineage progenitor (7–10). Expression of the transcription factors PU.1 and Ikaros in HSCs directs development away from the myeloid lineage and favors lymphocyte development (11, 12). Deficiency in either PU.1 or Ikaros results in a block to B cell development during transition from the MPP to CLP stage as well as the lack of expression of B-lineage-associated genes. Ikaros is crucial for expression of Flt3 in LMPPs (13–15) and likely plays a further role in differentiation of LMPPs and subsequent specification of the B cell fate (16). The transcription factors E2A, Ebf1, and Pax5 are required for specification of the B cell fate and transition from the CLP stage to the B-lineage-committed CD19⁺ pro-B cell (17, 18). Signals through Flt3 and PU.1 appear to upregulate expression of the IL-7R (19, 20). Although signaling through the IL-7R is not strictly required for formation of CLPs or pre-pro-B cells (21, 22), it appears to be required for maintaining B-lineage potential in CLPs and pre-pro-B cells augmenting expression of Ebf1 (22–24). E2A and Ebf1 lead to induction of Pax-5, which is required for expression of CD19 and maintaining commitment to the B cell lineage (25–28).

The Myb protooncogene (c-Myb) encodes a nuclear, DNA-binding protein that functions as both a transcription activator and repressor (29, 30). Expression of c-Myb has been primarily associated with hematopoietic tissue, although expression of Myb mRNA has been reported in other tissues (30). HSCs and progenitors of each hematopoietic lineage express c-Myb and the down-regulation of Myb expression is associated with hematopoietic maturation (31, 32). The pattern of Myb expression suggested that it plays a significant role in regulating hematopoiesis and this has been supported experimentally. Myb-null embryos develop normally to day 14, after which they die with severely disrupted patterns of erythroid and myeloid development (33). However, gaining insight into lineage-specific roles for c-Myb has been difficult due to the embryonic lethality of null Myb alleles. Normal B cell progenitors and mature B cells contain Myb mRNA and c-Myb protein, suggesting that c-Myb may play a role in B cell differentiation (34,
A role for c-Myb during B cell development was further supported by experiments that demonstrated Myb-null embryonic stem cells were unable to give rise to B-lineage cells in the Rag1−/− blastocyst complementation system (36). In addition, several hypomorphic mutants have been reported with reduced numbers of peripheral B cells and an apparent block in transition from the pro-B cell to the pre-B cell stage of differentiation (37–39). However, defective B cell development in these models could be due to defects in HSCs or very early progenitor cells.

We have previously used conditional inactivation of the Myb locus to demonstrate that c-Myb is crucial for transition from the pro-B cell to the pre-B cell stage of development and the maintenance of the pre-B cell compartment (34). Inactivation of the Myb locus in this study took place in pro-B cells, making it clear that c-Myb was important in B-lineage precursors. The pro-B to pre-B cell transition is particularly sensitive to the amount of c-Myb produced as Myb−/− heterozygotes are partially blocked at this stage of differentiation (40). Furthermore, pro-B cell to pre-B cell transition was disrupted by transgenic expression of the miR-150 microRNA (miRNA), which targets the Myb mRNA (40). Thus, different stages of B cell development appear to be exquisitely sensitive to differences in the amount of c-Myb produced.

We have now used cell-specific inactivation of the Myb locus in very early B-lineage progenitors with the Mbl-cre allele (41). The Mbl-cre allele leads to inactivation of a loxP-targeted Myb locus (MybΔ) beginning in the pro-B cell stage and is essentially complete in CD19−/− pro-B cells. We demonstrate that c-Myb is absolutely required for B cell development and MybΔ Mbl-cre progenitors failed to give rise to CD19−/− pro-B cells. We demonstrate that c-Myb plays a critical role in the survival of CD19−/− pro-B cells as well as proper expression of the α-chain of the IL-7R (CD127) and Ebf1. However, the survival defect in c-Myb-deficient CD19−/− pro-B cells appears to be independent of altered CD127 expression. Furthermore, development of CD19−/− B-lineage cells can be partially rescued from MybΔ/− Mbl-cre LMPPs by exogenously supplied Ebf1, suggesting that c-Myb regulates B cell development in part by controlling IL-7R-driven expression of Ebf1 as well as the survival of CD19−/− pro-B cells. Finally, we found that increased c-Myb expression in normal LMPPs can skew development of myeloid cells.

Materials and Methods

Mice

MybΔ/Δ, Myb−/−, CD19−/−, and Mbl-cre mice have been described previously (41–43). Rag2−/− mice (Taconic Farms) were bred at the University of Virginia. Mice were 8–12 wk old when used for experiments and were housed in a barrier mouse facility at the University of Virginia.

Abs and flow cytometry

Single-cell suspensions were prepared from 8- to 12-wk-old mice and 2 × 106 cells were stained with optimal amounts of fluorochrome-conjugated Abs as previously described (34). Cells were subsequently analyzed on a FACSCalibur (BD Immunocytometry Systems) or CyAn ADP (DakoCytomation). For intracellular staining, cell surface staining was performed on a FACSCalibur (BD Immunocytometry Systems) or CyAn ADP (DakoCyto) with 1 μM SYBR Green (Molecular Probes) and 0.4 μM of the primer set of interest in 25-μl reaction mixtures in an OptiCon DNA Engine (MJ Research). Conditions for quantitative RT-PCR were as follows: 95°C for 3 min, then 40 cycles of 95°C for 40 s, 66°C for 20 s, and 72°C for 30 s, followed by an extension at 72°C for 1 min. Melting curve analysis was then performed to ensure equivalent and appropriate melting temperatures. Each sample was normalized to the expression of Hprt (encoding hypoxanthine guanine phosphoribosyltransferase). Primers used were as follows: Myb forward, 5′-ATGCTGAGGACACCAA-3′; reverse, 5′-GGCGATCTTGTTCACCAAA-3′; and Hprt forward, 5′-GGCGAGTTGGAAAAAGTGTG-3′; reverse, 5′-CAGACGCCCCAGCAATGGTATG-3′.

Retroviral vectors

The retroviral vectors pMIG-R1, pMIG-EBF, pMIG-IL7Rα, and pMSCV-IRES-NGFR have been previously described (44, 47, 48). The retroviral vector pMIG-βc was a gift from Dr. T. J. Braciale (University of Virginia, Charlottesville, VA). To generate pMIG-c-Myb, a cDNA encoding a mouse c-Myb with an influenza hemagglutinin tag at the amino-terminal end was cloned into the BamHI/BglII site of pMIG-R1 (supplemental Fig. 51). To generate pMIG-Cre, Cre cDNA was isolated from pPGK-Cre-bpA and cloned into the XhoI/EcoRI site of pMIG-R1. To generate pntNGFR-Cre, Cre cDNA was cloned into the BglII/EcoRI site of pMSCV-IRES-NGFR. Retroviral supernatants were generated by transient transfection of HEK-293T cells and titered on NIH-3T3 cells by flow cytometry as previously described (49).

Cell culture

Pro-B cells from MybΔ/Δ Rag2−/−, MybΔ/Δ Rag2−/−, and MybΔ/Δ CD19−/− Rag2−/− mice were positively selected from bone marrow using CD19-labeled magnetic beads (Miltenyi Biotec). Cells were cultured in OptiMEM supplemented with 10% FBS (Life Technologies), 100 U/ml
Penicillin-streptomycin, 2 mM l-glutamine, and 50 μM 2-ME in flat-bottom 96-well plates, and total cells per well were analyzed 24, 48, and 72 h later by trypan blue exclusion. For transduction of Rag2⁻/− CD19⁻/⁺ pro-B cells, cells were selected on CD19-labeled magnetic beads, cultured for 24 h in Opti-MEM supplemented with 15% (v/v) FBS (Life Technologies), 5 ng/ml IL-7 (PeproTech), 100 U/ml penicillin-streptomycin, 2 mM l-glutamine, and 50 μM 2-ME and transduced with retroviral vectors as previously described (50). Following transduction, pro-B cells were cultured in Opti-MEM with 10% (v/v) FBS (Life Technologies), 100 U/ml penicillin-streptomycin, 2 mM l-glutamine, and 50 μM 2-ME with or without 5 ng/ml IL-7 and were analyzed 24, 36, and 48 h later by flow cytometry.

For transduction of LMPPs, LMPPs, defined as Lin⁻CD117⁺CD150high were electronically sorted from lineage-depleted bone marrow from Myb⁻/₀ and Myb⁻⁺/⁺ Mb1-cre mice and transduced as previously described (16). Following two successive rounds of retroviral transduction, cells were plated on OP9 stromal cells in Opti-MEM with 2.5% (v/v) FBS, 100 U/ml penicillin-streptomycin, 2 mM l-glutamine, and 50 μM 2-ME supplemented with 10 ng/ml stem cell factor (SCF; PeproTech), 10 ng/ml Flt3L (PeproTech), and 5 ng/ml IL-7 (PeproTech) and analyzed 10–14 days later by flow cytometry. Day 14.5 fetal liver progenitors were isolated following anti-CD24 complement-mediated lysis (Cedarlane Laboratories) as previously described (51). Following isolation, fetal liver progenitors were transduced and analyzed as described above.

Results

Expression of c-Myb mRNA in bone marrow B-lineage progenitor cells

We have previously used Myb⁻/₀ CD19⁻/⁺ cre mice to demonstrate that c-Myb is crucial for B cell development during transition from the pro-B cell to pro-B cell stage as well as for the maintenance of the pre-B cell compartment (34). However, although pre-B cells contain the greatest amount of Myb mRNA among bone marrow B-lineage subsets, HSCs and each progenitor stage of B cell development contain 10-fold more Myb mRNA than peripheral B cells, suggesting that c-Myb may be important for B lymphopoiesis before the pro-B to pre-B cell transition (Fig. 1). CD19 expression initiates in fraction B pro-B cells but deletion efficiency mediated by CD19-cre is incomplete in pro-B cells and is not detectable at earlier stages of B cell development, limiting its utility to assess c-Myb function earlier during B cell development (Refs. 34 and 41 and T. Bender, unpublished data). To circumvent this problem, we crossed Myb⁻/₀ mice to the Mb1-cre mouse strain to produce Myb⁻/₀ Mb1-cre mice. The Mb1-cre allele was created by replacing exons 2 and 3 of the Cd79a (Mb1) locus with a mammalian codon-optimized Cre recombine encoding cDNA, which has been reported to efficiently delete loxP-targeted DNA in CD19⁻/⁺ bone marrow B cells (41). To determine whether Mb1-cre also is able to delete in B-lineage progenitors before the pro-B cell stage, we electronically sorted HSCs, LMPPs, CLPs, and pre-pro-B cells as well as pro-B, pre-B, immature B, and recirculating B cells from Myb⁻/+ Mb1-cre mice and assessed the deletion efficiency of the Myb allele by PCR (Fig. 2). Cre-mediated deletion of the floxed Myb locus was barely detectable in HSCs, LMPPs, and CLPs. However, deletion of the floxed Myb allele was ~40% in pre-pro-B cells and was essentially complete in CD19⁻/⁺ pro-B cells and all subsequent stages of B cell development. Comparable results were obtained when the Mb1-cre allele was crossed onto the ROSA-floxed EYFP reporter mouse (data not shown). Thus, Mb1-cre provides earlier and more complete deletion of the Myb allele than CD19-cre.

c-Myb is required for the accumulation of CD19⁺ B cells in peripheral lymphoid tissue and bone marrow

Mb1-cre mice were crossed with Myb⁻/₀ to produce Myb⁻/₀ Mb1-cre mice. We initially compared the proportion of CD19⁺ B cells in the spleen, inguinal lymph nodes, and bone marrow of 8- to 12-wk-old Myb⁻/₀ Mb1-cre and control mice (controls are both Myb⁻/₀ and Myb⁻/+ Mb1-cre mice) by flow cytometry and found few, if any, B-lineage cells in each compartment (Fig. 3A). Similarly, the proportion of B1 B cells was severely reduced in peritoneal fluid from Myb⁻/₀ Mb1-cre compared with that of controls (Fig. 3B). The absolute number of B cells in the spleen was reduced >99% (Fig. 3C). In contrast, the proportion of CD4 and CD8 T cells in the spleen and inguinal lymph nodes was increased although the absolute number of splenic CD4 and CD8 T cells was reduced by ~70% and 50%, respectively (Fig. 3, C and D). However, the absolute number of thymocytes was not statistically different in thymi from Myb⁻/₀ Mb1-cre and control mice nor did we detect a difference in the distribution of CD4 and CD8 subsets in the thymi of mutant and control mice (Fig. 3, C and D). Cre-mediated deletion was extremely low in thymocytes (Fig. 2) and the reduced number of T cells detected in peripheral lymphoid tissues is consistent with the decreased number of peripheral T cells detected in other mouse models with severe defects in B lymphopoiesis that do not alter thymocyte development (52). Thus, in the absence of c-Myb, CD19⁺ B cells fail to accumulate in peripheral lymphoid tissue.
To better understand the drastic decrease between the number of peripheral B cells detected in Myb<sup>−/−</sup> Mbl-cre and control mice, we examined the distribution of bone marrow B-lineage subsets by flow cytometry (Fig. 4A). Few, if any, B220<sup>+</sup>IgM<sup>+</sup> B cells were detected in the bone marrow of Myb<sup>−/−</sup> Mbl-cre mice compared with controls. We previously identified a partial block during the pro-B to pre-B cell transition in Myb<sup>−/−</sup> CD19<sup>−/−</sup> mice that was manifested in part by a decreased number of fraction C pro-B cells (34) as defined by Hardy and colleagues (53). However, when we gated on B220<sup>+</sup>IgM<sup>+</sup> cells and examined the distribution of CD43 surface expression on cells from Myb<sup>−/−</sup> Mbl-cre and control bone marrow, we detected few, if any, B220<sup>+</sup>IgM<sup>+</sup>CD43<sup>−</sup> pro-B cells in Myb<sup>−/−</sup> Mbl-cre bone marrow. Furthermore, we failed to detect CD19<sup>+</sup> pro-B cells in Myb<sup>−/−</sup> Mbl-cre bone marrow (Fig. 4A). In contrast, we did not detect a statistically significant difference between the number of pro-pre-B cells (defined as B220<sup>+</sup>CD43<sup>+</sup>Ly6C<sup>−</sup>NK1.1<sup>−</sup>CD19<sup>−</sup>CD24<sup>−</sup>) in bone marrow from Myb<sup>−/−</sup> Mbl-cre compared with that of controls (Fig. 4, A and D). No difference was detected between the number of HSCs, LMPPs, or CLPs in bone marrow from Myb<sup>−/−</sup> Mbl-cre mice compared with that of controls (Fig. 4, B–D), which is in agreement with the lack of deletion detected at the floxed Myb locus in these cells (Fig. 2). Taken together, these results suggest that c-Myb is required for differentiation to the CD19<sup>+</sup> pro-B cell compartment, survival of the CD19<sup>+</sup> pro-B cell compartment, or both.

c-Myb is critical for the survival of CD19<sup>+</sup> pro-B cells

We have reported that Myb<sup>−/−</sup> CD19-cre and Myb<sup>+/−</sup> mice are impaired in pro-B to pre-B cell transition, yet the absolute number of pro-B cells is not decreased in these mice compared with Myb<sup>−/−</sup> mice, suggesting that c-Myb is not important for the maintenance of the pro-B cell compartment (34, 40). However, interpretation of these models is complicated by ongoing selection at the pre-BCR checkpoint. To separate maintenance of the pro-B cell compartment from events associated with selection at the pre-BCR checkpoint, we bred Myb<sup>−/−</sup> CD19-cre Rag2<sup>−/−</sup>, CD19<sup>−/−</sup> Rag2<sup>−/−</sup>, and Myb<sup>+/−</sup> Rag2<sup>−/−</sup> mice and characterized the CD19<sup>+</sup> pro-B cell compartment in each mouse strain. We detected no difference in the absolute number of pre-pro-B cells or CD19<sup>+</sup> pro-B cells between Myb<sup>−/−</sup> CD19-cre Rag2<sup>−/−</sup>, Myb<sup>+/−</sup> Rag2<sup>−/−</sup>, and control mice (Fig. 5A). However, surface expression of CD19 and CD24 was reduced on CD19<sup>+</sup> pro-B cells from Myb<sup>−/−</sup> CD19-cre Rag2<sup>−/−</sup> mice compared with Myb<sup>+/−</sup> Rag2<sup>−/−</sup> or Myb<sup>+/−</sup> Rag2<sup>−/−</sup> CD19<sup>+</sup> pro-B cells (Fig. 5B). The CD19-cre allele was made by replacing CD19 coding sequences with Cre, which results in a null allele (43). Thus, mice that carry a single CD19-cre allele have reduced expression of CD19 on all CD19<sup>+</sup> cells. However, reduced expression of CD24 is consistent with the notion that c-Myb- deficient pro-B cells either fail to express CD24 or fail to survive.
FIGURE 4. B cell development in Myb<sup>−/−</sup> Mb1-cre mice. A, Bone marrow from Myb<sup>−/−</sup> Mb1-cre and Myb<sup>−/−</sup> control mice was stained for surface expression of Ly6C, NK1.1, B220, IgM, CD43, CD19, and CD24. Viable cells were identified as DAPI<sup>−</sup>. The top tier represents B220 vs IgM after gating out Ly6C<sup>−</sup> and NK1.1<sup>−</sup> cells. Mature B cells were defined as Ly6C<sup>−</sup> NK1.1<sup>−</sup> B220<sup>+</sup>IgM<sup>+</sup> and immature B cells were defined as Ly6C<sup>−</sup> NK1.1<sup>−</sup> B220<sup>−</sup>IgM<sup>−</sup>. The middle tier represents B220 vs CD43 through a B220<sup>+</sup>IgM<sup>−</sup> gate. Pre-B cells were defined as Ly6C<sup>−</sup> NK1.1<sup>−</sup> B220<sup>−</sup>IgM<sup>−</sup>CD43<sup>−</sup>. The bottom tier represents CD19 vs CD24 through the B220<sup>−</sup>CD43<sup>−</sup> gate. Pro-B cells were defined as Ly6C<sup>−</sup> NK1.1<sup>−</sup> B220<sup>−</sup>IgM<sup>−</sup>CD43<sup>−</sup>CD19<sup>−</sup>CD24<sup>−</sup>. Data are representative of at least 10 mutant and control mice. B, Bone marrow was stained for surface expression of lineage markers (CD3<sup>−</sup>, CD11b, Ly6C, Ter119), CD19, B220, CD22, CD135, and CD127. Viable cells were identified as DAPI<sup>−</sup>. CLPs were defined as Lin<sup>−</sup> CD19<sup>−</sup> B220<sup>−</sup>CD127<sup>−</sup>CD135<sup>−</sup>. Data are representative of three mutant and three control mice. C, Bone marrow was stained for surface expression of lineage markers (B220, CD3<sup>−</sup>, CD11b, Ly6C, Ter119), CD19, CD22, CD135, and Sca1. Viable cells were identified as DAPI<sup>−</sup>. HSCs were defined as Lin<sup>−</sup> CD127<sup>−</sup>CD117<sup>−</sup>Sca1<sup>−</sup>. Data are representative of three mutant and three control mice. D, Absolute number of HSCs, CLPs, pre-pro-B cells, pro-B cells, pre-B cells, immature (Imm.) B cells, and mature (Mat.) B cells detected in Myb<sup>−/−</sup> Mb1-cre and Myb<sup>−/−</sup> mice. Data were compiled from three mutant and control mice. No statistically significant difference in HSCs, CLPs, and pre-pro-B cells between Myb<sup>−/−</sup> Mb1-cre mice and LMC. Asterisks (*) mark populations that are severely reduced in Myb<sup>−/−</sup> Mb1-cre mice.

To initially compare survival between Myb<sup>−/−</sup> CD19<sup>−/−</sup> Rag2<sup>−/−</sup>, Myb<sup>−/+</sup> Rag2<sup>−/−</sup>, and control Myb<sup>+</sup><sup>+/+</sup> Rag2<sup>−/−</sup> CD19<sup>+</sup> pro-B cells, we purified CD19<sup>+</sup> pro-B cells from each strain on anti-CD19-coated magnetic beads and placed them in liquid culture without an exogenous source of IL-7. Pro-B cells were harvested and viable cells were counted by trypan dye exclusion (Fig. 5C) or by flow cytometry (data not shown) after 24, 48, and 72 h of culture. We did not detect a statistically significant difference in the number of viable pro-B cells between strains at any time point during the experiment. However, we did detect a consistent decrease in the number of viable Myb<sup>−/−</sup> CD19<sup>−/−</sup> Rag2<sup>−/−</sup> pro-B cells after 24 and 48 h of culture compared with the other strains, although this did not reach statistical significance. To better assess the survival of c-Myb-deficient CD19<sup>+</sup> pro-B cells, CD19<sup>+</sup> pro-B cells were isolated from Myb<sup>−/−</sup> Rag2<sup>−/−</sup> mice on anti-CD19-coated magnetic beads and placed in liquid culture supplemented with IL-7 for 24 h. Subsequently, the pro-B cells were washed and transduced with retroviruses that produce Cre recombinase and GFP (MIG-Cre) or GFP alone (MIG-R1). After retrovirus transduction, the pro-B cells were placed back in liquid culture without exogenous IL-7 for 24, 48, and 72 h (Fig. 6A). Twenty-four hours after transduction, ≈40% of the cells that were transduced with either virus were GFP<sup>+</sup> as measured by flow cytometry. The proportion of GFP<sup>+</sup> and GFP<sup>−</sup> cells remained constant in pro-B cell cultures that were transduced with GFP alone over 72 h, suggesting that neither the transduced or untransduced cells had a growth advantage. In contrast, after transduction with MIG-Cre, only 10% of the viable pro-B cells were GFP<sup>+</sup> by 48 h after transduction, suggesting that the cells transduced with MIG-Cre failed to survive as well as the cells transduced with the MIG-R1 GFP-only producing virus. When the absolute number of viable cells was calculated, we found that the number of viable cells that were transduced with MIG-Cre rapidly decreased in culture compared with the number of cells transduced with MIG-R1 (Fig. 6A). Importantly, the number of viable Myb<sup>−/+</sup> Rag2<sup>−/−</sup> CD19<sup>+</sup> pro-B cells transduced with MIG-Cre did not decrease more rapidly in culture than untransduced cells (Fig. 6B). Thus, c-Myb is critical for the survival of CD19<sup>+</sup> pro-B cells.

To begin to gain insight into the basis for decreased pro-B cell survival in the absence of c-Myb, we initially assessed the surface expression of CD117 and CD127, which are cytokine receptors that are involved in maintaining the survival of pro-B cells, by flow cytometry. In particular, CD117 has been reported to be a target of c-Myb activity (54, 55). However, we did not detect a change in CD117 expression on Myb<sup>−/−</sup> Rag2<sup>−/−</sup> CD19<sup>+</sup> pro-B cells after transduction with a Cre/GFP-producing retrovirus (Fig. 7A). In contrast, surface expression of CD127 was decreased on Myb<sup>−/−</sup> Rag2<sup>−/−</sup> CD19<sup>+</sup> pro-B cells following transduction with Cre/GFP-producing retrovirus, suggesting that signaling through the IL-7R may be compromised. In addition, we found that mRNAs encoding CD127 and Ebf1 were decreased in c-Myb-deficient pro-B cells (Fig. 7B), but detected no change in expression of Bcl-x<sub>L</sub> or Mcl-1 as measured by flow cytometry (Fig. 7C) or RT-PCR (Fig. 7D). Furthermore, we did not detect decreased expression of Bcl-2 or Bim encoding mRNA (Fig. 7D). Decreased expression of CD127 in c-Myb-deficient pro-B cells may result in...
decreased survival signals. To test this notion, we isolated CD19<sup>+</sup> pro-B cells from Myb<sup>−/−</sup> Rag2<sup>−/−</sup> mice and transduced them with retroviruses that encode IL-7Rα/GFP (MIG-IL-7Rα), Ebf1/GFP (MIG-Ebf1), or c-Myc (MIG-cMYB) (Fig. 8A). The transduced cells were then cultured in IL-7 for 24 h and subsequently transduced with a second virus that encodes Cre and a truncated form of the nerve growth factor receptor that is unable to signal (tNGFR-Cre). The cells were then placed in liquid culture with or without IL-7 and survival of the cotransduced cells (identified by coexpression of GFP and tNGFR using flow cytometry) was assessed after 24 and 48 h of culture. c-Myc was able to rescue the survival of CD19<sup>+</sup> cells in liquid culture lacking IL-7 (Fig. 8A). However, survival was not rescued with exogenous CD127 or survival of CD19<sup>+</sup> pro-B cells from Myb<sup>−/−</sup> Rag2<sup>−/−</sup> mice transduced with tNGFR-Cre or tNGFR-Cre plus MIG-R1 decreased by ~65% between 24 and 48 h of culture. Similarly, the recovery of CD19<sup>+</sup> pro-B cells from Myb<sup>−/−</sup> Rag2<sup>−/−</sup> mice transduced with tNGFR-Cre plus MIG-IL-7Rα also decreased by ~65% between 24 and 48 h of liquid culture supplemented in the presence of IL-7, demonstrating that c-Myc-deficient CD19<sup>+</sup> pro-B cells fail to survive in the presence of IL-7 even when provided with exogenous supplied IL-7Rα. In addition, CD19<sup>+</sup> pro-B cells transduced with control virus (tNGFR) or a Cre-producing virus (tNGFR-Cre) plus MIG-R1 or MIG-IL-7Rα were stained with DRAQ5 and the DNA content was measured by flow cytometry (Fig. 8C). Populations of cells that were transduced with tNGFR-Cre plus MIG-R1 or MIG-IL-7Rα contained 5- to 7-fold more cells with a <2n DNA content than cells transduced with tNGFR plus MIG-R1 or
MIG-IL-7Rα. Thus, decreased viability of c-Myb-deficient pro-B cells is not simply due to reduced expression of CD127 or Ebf1.

Ebf1 can partially rescue production of CD19+ cells from Mybf/f Mb1-cre progenitor cells

Transition from the pre-pro-B cell to the CD19+ pro-B cell compartment is dependent on signals through the IL-7R, which leads to increased expression of Ebf1 and differentiation to the CD19+ pro-B cell compartment (22–24). Decreased expression of CD127 and Ebf1 after deletion of the floxed c-Myb alleles suggests that c-Myb may also be important for transition from the pre-pro-B cell to the CD19+ pro-B cell compartment in addition to survival of CD19+ pro-B cells. However, we did not detect a difference in expression of CD127 between Mybf/f Mb1-cre and control pre-pro-B cells (Fig. 9A), which could have been due to incomplete deletion at the Myb locus in pre-pro-B cells from Mybf/f Mb1-cre mice (Fig. 2) or that c-Myb-deficient pre-pro-B cells have poor viability and die in vivo before we could detect a decrease in CD127 expression. LMPPs from Mybf/f Mb1-cre and control mice were electronically sorted and plated onto OP9 stromal cell cultures supplemented with SCL, Flt3L, and IL-7 to determine whether they could generate CD19+ cells (Fig. 9, B and C). The vast majority of cells that grew in cultures derived from control LMPPs were CD19+ B-lineage cells while very few CD19+ cells grow out of the Mybf/f Mb1-cre LMPPs (Fig. 9C). Although very few CD19+ cells grow out of the Mybf/f Mb1-cre LMPPs in these cultures compared with control LMPPs, there is not an appreciable increase in the number of Gr1+ or CD19+ Gr1− cells that grow out of these cultures. Thus, the change in proportion of cells that grow out of the Mybf/f Mb1-cre mice compared with controls. To determine whether decreased signals through the IL-7R could explain the lack of CD19+ pro-B cells in Mybf/f Mb1-cre mice, LMPPs were electronically sorted from Mybf/f Mb1-cre and control bone marrow transduced with MIG-R1, MIG-cMYB, MIG-IL-7Rα, or MIG-Ebf1 and subsequently cultured for 14 days on OP9 stromal cells as described above. Few CD19+ B-lineage cells grew out of the cultures transduced with MIG-R1-, MIG-cMYB-, or MIG-IL-7Rα-producing retroviruses (Fig. 9D). Furthermore, transducing Mybf/f Mb1-cre LMPPs with a Bcl-xL-producing retrovirus also

**FIGURE 7.** Reduced expression of Il7r and Ebf1 mRNA in the absence of c-Myb. A, Surface expression of CD117 (c-kit) and CD127 (IL-7Rα) on Mybf/f Rag2−/− CD19+ pro-B cells transduced with MIG-R1 or MIG-Cre and analyzed 24, 36, and 48 h after transduction. Viability cells were identified as 7-aminoactinomycin D−. B, Relative expression of Il7r and Ebf1 mRNA in Mybf/f Rag2−/− CD19+ pro-B cells transduced with tNGFR or tNGFR-Cre was measured by semiquantitative RT-PCR 24, 36, and 48 h after transduction and normalized to the expression of ActB. Transduced cells were isolated after treatment with biotinylated anti-tNGFR Ab and streptavidin-coated magnetic beads before making RNA. C, Intracellular expression of Bcl-xL, McI1, and Bim performed on 3-fold serial dilutions of cDNA prepared from Mybf/f Rag2−/− CD19+ pro-B cells transduced with tNGFR or tNGFR-Cre 24 and 36 h after transduction. ActB serves as a loading control.
was not able to rescue differentiation of CD19+ pro-B cells (supplemental Fig. 2), suggesting that survival is not the sole basis for the severe deficit in the accumulation of CD19+ pro-B cells in Myb<sup>−/−</sup> Mb1-cre mice. However, LMPPs transduced with MIG-Ebf1 produced an increased number of CD19+ B-lineage cells, representing a >20-fold stimulation over cultures transduced with GFP or IL-7Rα/GFP, suggesting that bypassing the need for signals through the IL-7R by overexpression of Ebf1 can at least in part overcome c-Myb deficiency to promote transition from the pre-pro-B cell to the CD19+ pro-B cell compartment. However, the number of CD19+ pro-B cells that grow out in OP9 stromal cell culture from MIG-Ebf1-transduced Myb<sup>−/−</sup> Mb1-cre LMPPs is still only 10–15% of the number CD19+ cells that grow from control LMPPs (cf Fig. 9, C and D), suggesting that Ebf1 can only provide a partial rescue of B-lineage development. This is similar to the finding that Ebf1 can rescue differentiation of IL-7R- or E2A-deficient B-lineage progenitors but not proliferative expansion (22, 56, 57).

Surprisingly, c-Myb and CD127 failed to rescue production of CD19+ cells from Myb<sup>−/−</sup> Mb1-cre LMPPs. When we more closely examined the cells that grew out from Myb<sup>−/−</sup> Mb1-cre or control LMPPs transduced with MIG-cMYB, we found that in both cases c-Myb appeared to favor the production of Gr1+ rather than CD19+ producing cells (Fig. 9E), suggesting that increased c-Myb expression in LMPPs may block differentiation to the B cell lineage or favor the growth of myeloid cells. We similarly tried to rescue CD19+B-lineage cell development from Myb<sup>−/−</sup> fetal liver progenitor cells. In this case, we were unable to rescue growth of CD19+ or Gr1+ cells with MIG-R1, MIG-cMYB, MIG-IL-7Rα, or MIG-Ebf1 (data not shown). However, transduction of control fetal liver progenitors with MIG-cMYB resulted in the outgrowth of almost entirely Gr1+ cells (Fig. 9F). Thus, proper control of c-Myb expression appears to be crucial in determining the growth of myeloid vs B-lineage growth from LMPPs.

**Discussion**

Several studies have suggested that c-Myb plays an important role during B lymphopoiesis. First, B cells failed to develop in Rag1<sup>−/−</sup> blastocyst chimeras made with Myb<sup>−/−</sup> mouse embryonic stem cells (36). Second, a reduced number of peripheral B cells has been identified in several hypomorphic c-Myb mutants (37–39). However, in each case, the decrease in peripheral B cell number could be related to defects in HSCs or early progenitors. We recently used Myb<sup>−/−</sup> CD19-cre mice to identify a partial block in B cell development during the pre-pro-B to pre-B cell transition (34). In addition, peripheral B cells in these mice were hyporesponsive to BLyS. CD19<sup>−/−</sup>directs deletion of floxed Myb alleles relatively late in the pro-B cell compartment, making it difficult to determine whether c-Myb is important at earlier stages of B cell development (34, 41, 43). Most recently, we found that forced expression of the miR-150 miRNA, which targets the Myb mRNA, resulted in a partial block to B cell development during the pre-B to pre-B cell transition that was associated with cell death when B-lineage cells were grown in stromal cell cultures supplemented with IL-7 (40). This study made clear that relatively small differences in c-Myb expression can have a significant impact on B cell development. However, by breeding Myb<sup>−/−</sup> Mb1-cre mice, where inactivation of the Myb locus occurs earlier than in CD19-cre mice but specifically in the B lineage, we have demonstrated that c-Myb is absolutely required for B cell development. The severe deficit in B cell development detected in Myb<sup>−/−</sup> Mb1-cre mice demonstrates that c-Myb must be considered along with E2A, Ebf1, and Pax5 as a transcription factor that is critical for B cell development and is included in the regulatory network that controls B cell development.

Mb1-cre-mediated deletion is initiated in pre-pro-B cells. Thus, the failure to produce CD19+ pro-B cells could be due to a failure of developing B cells to survive, failure to differentiate beyond the pre-pro-B cell stage, or both. We have previously reported that B cell development is partially blocked during transition from the pre-B cell to the pre-B cell compartment (34). Furthermore, mice that are transgenic for the miR-150 miRNA, which targets c-Myb mRNA, have a similar block during B cell differentiation (40). Enriched B-lineage cells from miR-150-transgenic mice that are grown in stromal cell culture with IL-7 accumulate 2- to 3-fold
more dead cells than cells from control mice, suggesting that prolif-erating pro-B or pre-B cells undergo apoptotic cell death. Cell death in this experiment could have been due to events associated with pre-BCR selection. Although it seemed unlikely that pre-BCR-mediated events could explain the severe deficit of CD19^{-} B cells in Mybf^{-/} Mb1-cre mice, we crossed Myb^{+/} and Mybf^{-/} CD19^{-} pre-B cells with Rag2^{−/−} mice to separate events associated with V(D)J recombination and the pre-BCR checkpoint from the intrinsic survival of pro-B cells. We did not detect a difference in the number of pre-pro-B cells or CD19^{+} pro-B cells in Myb^{+/−} or Mybf^{-/} CD19^{-} pre-B cells with Rag2^{−/−} mice to separate events associated with V(D)J recombination and the pre-BCR checkpoint from the intrinsic survival of pro-B cells. We did not detect a difference in the number of pro-B cells from control mice compared with pre-BCR selection.

To gain insight into how c-Myb might mediate survival, we assessed expression of several genes that are associated with survival in pro-B cells, including Il7r and Mcl1. Mice deficient for Il7r or Mcl1 have blocks to B cell development that are very similar to those of Mybf^{-/} Mb1-cre mice (22, 24, 58, 59). Inactivation of the Myb locus did not result in decreased expression of CD117, which is thought to be a direct c-Myb target in some systems (55, 60), Bcl-xL, Mcl-1, Bcl-2, or Bim. Both CD127 surface expression and mRNA were decreased after Cre-mediated inactivation of the Myb locus in CD19^{+} pro-B cells, suggesting that c-Myb might control pro-B cell viability by regulating expression of CD127. However, transduction of CD19^{+} pro-B cells with CD127 before inactivation of the Myb locus did not rescue survival of the pro-B cells. Thus, c-Myb-dependent survival of CD19^{+} pro-B cells appears to be mediated independent of CD127 expression. How c-Myb is involved in regulating expression of CD127 remains unknown. The CD127 promoter region contains two potential c-Myb binding sites but we have been unable to demonstrate direct binding of c-Myb to the CD127 promoter using a chromatin immunoprecipitation assay (S. P. F. and T. P. B., unpublished data). Thus, c-Myb may be involved in regulating CD127 expression by an indirect mechanism. It remains possible that c-Myb may interact with unidentified regulatory regions that control expression of CD127 or by interacting with other proteins that may tether c-Myb to the CD127 promoter and, in this respect, c-Myb has been reported to regulate transcription of the vascular endothelial growth factor promoter in a DNA-binding domain independent fashion (61). Further work is required to understand how c-Myb regulates expression of CD127.

Inactivation of the Myb locus in CD19^{+} pro-B cells resulted in decreased surface expression of CD127 as well as decreased Il7r and Ebf1 mRNA. Specification of the B cell fate in CLPs and transition from the pre-pro-B cell stage to the CD19^{+} pro-B cell stage is dependent on signals through the IL-7R that result in increased expression of Ebf1 (22–24). Thus, c-Myb may be required for proper CD127 expression before the CD19^{+} pro-B cell stage. LMPPs isolated from Mybf^{-/} Mb1-cre mice produced <0.5% of the number of CD19^{+} cells produced by LMPPs from control mice.

**FIGURE 9.** Ebf1 can partially rescue B-lineage development from Myb^{−/−} Mb1-cre LMPPs. A. Surface expression of CD127 on pre-pro-B cells, defined as Ly6C^{−} NK1.1^{−} B220^{−} CD43^{+} CD19^{−} CD24^{−}, from Mybf^{-/} Mb1-cre and Myb^{+/−} mice compared with FMO control. B. Five thousand LMPPs from Myb^{+/−} and Myb^{−/−} Mb1-cre mice were seeded on OP9 stromal cells in the presence of SCF, Flt3L, and IL-7 and analyzed 14 days later by flow cytometry for surface expression of CD19 and Gr1. Bars represent percentage of positive cells from each culture. Data are representative of four independent experiments. C. Total number of CD19^{+}, Gr1^{+}, and CD19^{−} Gr1^{+} cells that grew out of OP9 stromal cell co-culture with Myb^{+/−} and Myb^{−/−} Mb1-cre LMPPs. Data are representative of four independent experiments. D. LMPPs were isolated from Myb^{+/−} Mb1-cre bone marrow, seeded at 5000 cells/well on OP9 stromal cells and transduced with MIG-R1, MIG-cMYB, MIG-IL-7Rα, or MIG-Ebf1. LMPPs were cultured in the presence of SCF, Flt3L, and IL-7 and analyzed 14 days later by flow cytometry for expression of B220 and CD19 and the number of GFP^{+} B220^{+} CD19^{−} cells were determined. Data are representative of four independent experiments. E. Myb^{+/−} and Myb^{−/−} Mb1-cre LMPPs were transduced with MIG-R1 or MIG-cMYB and cultured on OP9 stromal cells in the presence of SCF, Flt3L, and IL-7. Cultures were analyzed 14 days later by flow cytometry for surface expression of CD19 and Gr1. Numbers in the quadrants indicate percent cells in each. Data are representative of four independent experiments. F. Day 14.5 fetal liver progenitors from Myb^{+/−} embryos were transduced with MIG-R1 or MIG-cMYB and cultured on OP9 stromal cells in the presence of SCF, Flt3L, and IL-7. Cultures were analyzed 14 days later by flow cytometry for the surface expression of CD19 and Gr1. Numbers in the quadrants indicate percent cells in each.

![Figure 9](http://www.jimmunol.org/DownloadedFrom)
when grown on OP9 stromal cells. Thus, the phenotype of Mybf/f Mbl-cre mice could be replicated in vitro. To determine whether c-Myb, CD127, or Ebf1 could rescue development to the CD19+ pro-B cell stage, we transduced LMPPs from Mybf/f Mbl-cre mice with retroviruses encoding c-Myb/GFP, CD127/GFP, Ebf1/GFP, or GFP alone. Surprisingly, we found that neither c-Myb nor CD127 could rescue differentiation of Mybf/f Mbl-cre LMPPs. Proper levels of c-Myb expression have been found to be a crucial determinant of hematopoietic differentiation in a variety of normal and leukemic models of hematopoiesis (37, 62–64). One study that used a tetracycline-inducible c-Myb allele reported that overexpression of c-Myb could block differentiation of erythroid, megakaryocytic, and B lymphocyte development while myeloid development was not inhibited (62). We found that transduction of Mybf/f LMPPs or Myb+/− fetal liver progenitors with c-Myb/GFP appeared to skew differentiation of normal LMPPs away from B cell development in OP9 stromal cell cultures and toward myeloid development. It is likely that expression of exogenous c-Myb in cells that already produce normal amounts of c-Myb directs differentiation away from or blocks B-lineage development but allows myeloid development to proceed. Similarly, overexpression of CD127 has previously been reported to block B cell development although the basis for this is not understood (65).

Transduction of Mybf/f Mbl-cre LMPPs with MIG-Ebf1 resulted in a 20-fold increase in the number of CD19+ cells produced compared with LMPPs transduced with MIG-R1. This represented a 20-fold increase over Mybf/f Mbl-cre LMPPs transduced with GFP alone, suggesting that c-Myb may be important for expression of CD127 before the CD19+ pro-B cell stage, which in turn is needed for increased expression of Ebf1 and transition to the CD19+ pro-B cell compartment. The finding that Ebf1 can partially rescue differentiation of Mybf/f Mbl-cre pro-B cells to CD19+ cells suggests that c-Myb is not required for commitment to the B cell lineage. Thus, c-Myb appears to play at least two important roles during B cell development. First, c-Myb appears to the B cell lineage. Thus, c-Myb appears to play at least two roles in the B cell compartment, suggesting that two Myb alleles are required at this stage of B cell development. It will be important to identify direct targets of c-Myb activity at different stages of B cell development to understand the basis for c-Myb activity during B lymphopoiesis.

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Disclosures
The authors have no financial interest of interest.

References


SUPPLEMENTAL FIGURE 1. Expression of HA-tagged c-Myb from pMIG-cMYB. HEK 293T cells were transiently transfected with pMIG-R1 or pMIG-cMyb by transient calcium phosphate transfection. Cells were lysed 24 hours later in 20 mM Tris pH 7.4, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Triton X-100 containing EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). Fifty micrograms of protein was fractionated on 10% SDS-polyacrylamide gel and transferred to Protran nitrocellulose transfer membranes (Whatman, Dassel, Germany). Membranes were blocked in PBS + 0.05% Tween-20 (PBS-T) with 5% non-fat dry milk for 1 hour and then incubated overnight at 4°C with either anti-c-Myb (clone 1-1, Millipore, Bedford, MA) or anti-HA (clone Y11, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed three times in PBS-T and probed with either anti-rabbit-HRP or anti-mouse-HRP conjugated antibody in PBS-T for 1 hour at room temperature. After washing the membrane three times in PBS-T, the proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ). Asterisk (*) represents a nonspecific interaction that serves as a loading control.
SUPPLEMENTAL FIGURE 2. Overexpression of Bcl-xL fails to rescue B-lineage development from Myb<sup>f/f</sup> Mb1-cre LMPPs. LMPPs were isolated from Myb<sup>f/f</sup> Mb1-cre bone marrow by fluorescence activated cell sorting, seeded at 5000 cells per well on OP-9 stromal cells and transduced with MIG-R1, MIG-BclxL or MIG-Ebf1. LMPPs were cultured in the presence of SCF, Flt3L, and IL-7 and analyzed 10 days later by flow cytometry for the expression of B220 and CD19 and the number of GFP+ B220+ CD19+ cells were determined.