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

Mireia Vallespinós, David Fernández, Lorena Rodríguez, Josué Alvaro-Blanco, Esther Baena, Maitane Ortiz, Daniela Dukovska, Dolores Martínez, Ana Rojas, Miguel R. Campanero and Ignacio Moreno de Alborán

J Immunol 2011; 186:6726-6736; Prepublished online 13 May 2011;
doi: 10.4049/jimmunol.1002753
http://www.jimmunol.org/content/186/12/6726

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/05/13/jimmunol.1002753.DC1

References
This article cites 55 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/186/12/6726.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/PUBLICATIONS/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
B Lymphocyte Commitment Program Is Driven by the Proto-Oncogene c-myc

Mireia Vallespinós,*1 David Fernández,*1 Lorena Rodríguez,*1 Josué Alvaro-Blanco,† Esther Baena,*2 Maitane Ortiz,* Daniela Dukovska,* Dolores Martínez,† Ana Rojas,‡ Miguel R. Campanero,† and Ignacio Moreno de Albornoz*1

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 transcription factors E2A, EBF-1, and Pax-5 play 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\textsubscript{α\textalpha}-to-D\textsubscript{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\textsuperscript{-/-} 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\textsubscript{0}/G\textsubscript{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

*Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología/Consejo Superior de Investigaciones Científicas, E-28049 Madrid, Spain; †Instituto de Investigaciones Biomédicas/Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, E-28049 Madrid, Spain; and ‡Centro Nacional de Investigaciones Oncológicas, E-28029 Madrid, Spain

1M.V., D.F., and L.R. contributed equally to this work.

2Current address: Division of Hematology/Oncology, Children’s Hospital Boston, Harvard Medical School, Boston, MA.

Received for publication August 13, 2010. Accepted for publication April 11, 2011.

This work was supported by grants (to I.M.A.) from the Spanish Ministry of Science and Innovation (SAF2008-00118), the Mutua Madrileña Foundation, the Concern Foundation, and the Madrid regional government (S-SAL-0304-2006). M.V. is the recipient of a fellowship from the Tecnomambiente Company, D.F. and E.B. are the recipients of fellowships from the Spanish Ministry of Science and Innovation, M.O. is supported by a fellowship from the Madrid regional government and Fondo Social Europeo, and D.D. is the recipient of a “La Caixa” Foundation fellowship.

Address correspondence and reprint requests to Dr. Ignacio Moreno de Albornoz, Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología/Consejo Superior de Investigaciones Científicas, Darwin 3, Cantoblanco, E-28049 Madrid, Spain. E-mail address: moreno@cnb.csic.es

The online version of this article contains supplemental material.

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

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002753
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<sup>fl/fl</sup>;mx<sup>cre</sup> mice was described (28). To generate c-myc<sup>fl/fl;mb1<sup>cre</sup> mice, c-myc<sup>fl/fl</sup> mice were bred with mb1<sup>cre</sup> mice (29), and progeny were crossed to yield homozygous (c-myc<sup>fl/fl;mb1<sup>cre</sup></sup>) and control mice (c-myc<sup>fl/fl;mb1<sup>cre</sup></sup> or c-myc<sup>fl/fl;mb1<sup>fl/fl</sup></sup>). c-myc<sup>fl/fl;mb1<sup>cre</sup></sup> mice were bred with ikneo<sup>+</sup> mice (30) to generate ikneo<sup>+</sup>;c-myc<sup>fl/fl</sup>;mx<sup>cre+</sup> and ikneo<sup>+</sup>;c-myc<sup>fl/fl</sup>;mb1<sup>cre+</sup> mice, respectively. Progeny were crossed to generate homozygous (ikneo<sup>+</sup>;c-myc<sup>fl/fl</sup>;mx<sup>cre+</sup> or ikneo<sup>+</sup>;c-myc<sup>fl/fl</sup>;mb1<sup>cre+</sup>) mice. Mice were genotyped using a PCR-based analysis of tail genomic DNA (28). Primers for c-myc<sup>fl/fl</sup>;mx<sup>cre+</sup>;ikneo/+;c-myc<sup>fl/fl</sup>;mx<sup>cre+</sup>;ikneo/+;c-myc<sup>fl/fl</sup>;mb1<sup>cre+</sup>;ikneo/+;c-myc<sup>fl/fl</sup>;mb1<sup>fl/fl</sup>;ikneo/+;c-myc<sup>fl/fl</sup>;mb1<sup>cre+</sup>;ikneo/+;c-myc<sup>fl/fl</sup>;mb1<sup>fl/fl</sup>;ikneo/+;c-myc<sup>fl/fl</sup>;mb1<sup>cre+</sup>;ixneito<sup>+</sup>alleles (mb1in1 and mb1in2). The knock-in allele (ikneo<sup>+</sup>) was identified as described (30). c-myc<sup>fl/fl;mx<sup>cre+</sup></sup> or c-myc<sup>fl/fl;mb1<sup>cre+</sup></sup> mice were bred with rosa26<sup>GFP</sup> mice. The rosa26<sup>GFP</sup> allele was genotyped as described (31).

**Polyniosinic-polycytidyl acid injections**

To induce c-myc deletion in c-myc<sup>fl/fl;mx<sup>cre+</sup></sup> and ikneo<sup>+</sup>;c-myc<sup>fl/fl;mx<sup>cre+</sup></sup> mice, 4- to 6-wk-old animals received three i.p. injections of polyniosinic-polycytidyl acid (pPc; 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 progeny (Ly-6c<sup>-</sup>;NK1.1<sup>-</sup>); B220<sup>-</sup>CD25<sup>+</sup>IgM<sup>+</sup> and B220<sup>-</sup>CD25<sup>-</sup>IgM<sup>+</sup>) from total BM suspensions with streptavidin Dynabeads (Invitrogen) by incubation with a mixture of biotinylated Abs to lineage markers (B220, CD4, CD8, Ter119, Gr1, and CD11b; all from BD Pharmingen). Cells were cultured at a concentration of 10<sup>6</sup> 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 ILF (10<sup>3</sup> U/ml). Stimulated Lin<sup>-</sup> 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, 0.003% w/v Primo- ratone LM (Sigma-Aldrich), and 50 mM 2-MA 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.

**V(D)J recombination analysis**

Genomic PCR amplification of Ig genes was performed with V<sub>i</sub>-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 V<sub>i</sub>3558-J<sub>3</sub> 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 (5′-GATACAGCGAAGATGTCCCTC, antisense (5′-GGGTTGGTGATGATGTAAG). For c-myc, cd19 (5′-ATACAGCGAAGATGTCCCTCA, c-myc for 5′-CTCAAGGTCGCAACTGTCTG-3′) for ef3a, Ef1a (5′-CTATGCTTCGCTCATTGAAAAAAG) and Ef1b-1 (5′-CATGACTCTGTCCTGTTGGA3′) were lncDNA (10-fold dilution series, 4 h). Medium was replaced with IMDM containing 2% heat-inactivated FBS, 1 mM L-glutamine, 1 mM penicillin/streptomycin, 0.003% w/v Primo ratone LM (Sigma-Aldrich), and 50 mM 2-MA 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.

**Luciferase assay activity**

The murine ef1b-1 α promoter was amplified by PCR using the primers Ebf1a<sub>5</sub>-<sub>10</sub> (5′-TAAGACGCCGAACTTGCTC-3′) and Ef1b<sub>3</sub>-<sub>10</sub> (5′-ATGCTTGGAGCTCCTGCTAGAAAT-3′) for ef1a, Ef1b-1 (5′-CATGACTCTGTCCTGTTGGA-3′) for cd19, Ef1a (5′-ATACAGCGAAGATGTCCCTCA-3′) and Ef1b-1 (5′-CTATGCTTCGCTCATTGAAAAAAG) and Ef1b-1 (5′-CATGACTCTGTCCTGTTGGA-3′) for cd19, Ef1a (5′-ATACAGCGAAGATGTCCCTCA-3′) and Ef1b-1 (5′-CATGACTCTGTCCTGTTGGA-3′) for cd19. Control vector (Promega) upstream of the luciferase gene to generate the vector pGL3-Ebf1a. All of the constructs were sequenced. For luciferase assays, HEK 293T cells were cultured in 24-well plates and cotransfected with 500 ng pGL3-Ebf1a or pGL3-Control vector and increasing amounts of pRVR-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 crosslinked with formaldehyde (1% final concentration) and incubated (room temperature, 20 min). Rabbit polyclonal anti-c-Myc (Santa Cruz Biotechnology) or preimmune serum was used to precipitate chromatin from 2 × 10<sup>6</sup> 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 a<sub>input</sub>/a<sub>input</sub>) using the primers flanking E-box5 were: EFS-FW (5′-CCTCCAGCTCTTGAGAAGG-3′) and EFS-REV (5′-ACTCCAGAAGGTTAGAACC-3′).

**EMSA**

Assays were performed with labeled double-stranded oligonucleotides encompassing E-boxes from the dbf7 and dbf1 α promoters. pcDNA3-c-
Myeloid and pDNA3-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-7648) or 1 μg anti-ε-Myc (sc-517a) Abs (both from Santa Cruz Biotechnology) were used. Double-stranded oligonucleotides were used: DHFR-F-wt (5‘-GGGCCGACACCGTTGGGCTC-3‘), DHFR-F-mut (5‘-GGGCCGACACCGTTGGGCTC-3‘), EBS-wt (5‘-GGTCTCTACCCCGTTACGTCGCT-3‘), and EBS-mut (5‘-GGTCTCTACCCCGTTACGTCGCT-3‘), EBS-exon (5‘-GGTCCTACCCCGTTACGTCGCT-3‘), and EBS-intron (5‘-GGTCCTACCCCGTTACGTCGCT-3‘), DHFR-I-wt (5‘-GGCGCGACACCCACGTGCCCT-3‘), DHFR-I-mut (5‘-GGCGCGACACCCACGTGCCCT-3‘) were used. Double-stranded oligonucleotides were: 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘ and 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘, 5‘-GTCCAAGGCAGTCAGGAAGTTA-3‘ (c-Myc), 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘ (c-myc), 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘ (c-myc), 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘ (c-myc). Double-stranded oligonucleotides were: 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘, 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘, 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘, 5‘-GAGACTGCAGTCAACGTGGGTAGGA-3‘.

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 (29), cre-exon transgenic (36), and rosa26resflox reporter mice (31). In mb1-fl/fl mice, the mb1-cre allele is expressed from the earliest stage of B lymphocyte differentiation (29). In mb1-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 rosa26resflox 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). 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+IgM+) in mb1-fl/fl mice 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 34-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. Analysis of Hardy fractions showed a decrease in the absolute numbers from fractions A to E (Fig. 1F, 1G, Supplemental Fig. 2E–G). Fraction F seems to be less affected, which is consistent with our previous results in mature B cells (21) (Fig. 1F, 1G). Annexin V staining showed an increase (35.8 versus 16.2%) 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. 2B). Recirculating mature B lymphocytes (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(+/-)cd19(+/-) mice (21), indicating that c-myc is dispensable for mature B lymphocyte maintenance (Figs. 1F, 2B). 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 mb1-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 61.6% 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(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 impaired Ig gene recombination, we analyzed V(D)J recombination by genomic PCR in sorted pro- and pre-B cells. We did not observe significant differences in D1H-JH and V1Hproximal and VD1Hdistal to D4HJ 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 V1Hproximal (1.9-fold) and VD1Hdistal to D4HJ 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(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 tfce2a, ebf-1, ikars, 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-fl/fl mice. (Fig. 4A). Inter-
estingly, tcf2a−/−, 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. 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−/+ 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 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. 4A). Similarly, pro- and pre-B cells from gfp-nx-fl/fl mice showed reduced cd79a, cd79b, and pax-5 expression (Supplemental Fig. 4B). 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−CD25−IgM+).
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 β 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 (2.5-fold) and in the L1-2 B cell line (2-fold) (Fig. 5A–C). Luciferase assays showed that site-directed mutagenesis of the E-box5 (EBA5), 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. 5A–C). c-Myc-dependent transactivation was not observed with genomic regions containing the pax-5 or tcf2a promoters (Supplemental Fig. 5C, 5D).

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-
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_{H2}J_{H2} 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 tcf2a2a (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° 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, 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° versus 0.1 × 10°) 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 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 BM B lymphocytes. c-myc transgenic (Eμ-c-myc) and rag-1°/° mice were included for comparison. Data represent at least three independent experiments.

---

The Journal of Immunology 6731
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 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− 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, 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 tcf7l2a (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>+/−</sup> 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>mbl</i>-<i>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 <i>mx-fl/fl</i> with <i>E<sub>m</sub>-bcl-2</i> 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 <i>ik-mb1-fl/fl</i> 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 <i>ik-mx-fl/fl</i> 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 <i>ik-mx-fl/fl</i> 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 <i>ik-mx-fl/fl</i> and control mouse BM. B, Absolute numbers of B cell subpopulations in BM of mice in A. <i>ik-mx-fl/fl</i>, n = 5; <i>fl/fl</i>, n = 6; <i>mx-fl/fl</i>, n = 4. C, c-myc deletion in sorted populations from mice of the indicated genotypes. Data represent two independent experiments. **<i>p</i> < 0.01.
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 during B lymphocyte differentiation such as cell survival or proliferation. The capacity of c-Myc to regulate these functions during B lymphocyte differentiation such as cell survival or proliferation.

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.

Acknowledgments

We thank I. Antón, C. Cobaleda, D. Martínez, and L. Torroja for a critical reading of the manuscript. M.A.R. Marcos for his input, M. Busslinger for reading of the manuscript, M.A.R. Marcos for his input, M. Busslinger for reading of the manuscript, M. Reth for mb1-cre mice, H. Singh for B220-MIG vectors, the Centro Nacional de Biotecnología animal facility and the Departamento de Immunología y Oncología and Centro Nacional de Investigaciones Oncológicas FACS facility for help, and M. Mark for editorial assistance. Bi-otin-anti-NK1.1 Ab was a gift from C. Ardavin. This article is dedicated to the memory of M.A.R. Marcos.

Disclosures

The authors have no financial conflicts of interest.

References


Fig. S1. Deletion of c-myc and induction of GFP expression in gfp-mb1-fl/fl and heterozygous gfp-mb1-fl/+ mice. (A) Outline of breedings to generate the indicated mouse models. (B, C) Flow cytometry analysis of BM and spleen cells from mice of the indicated genotype. Experiment representative of at least three independent experiments. (D) Genomic PCR analysis of wt, deleted and flox alleles of c-myc from sorted B220+IgM+ BM cells from the mice shown in (B). Numbers indicate individual mice. Experiment representative of two independent experiments with a total of 4 mice for each genotype. (E) Genomic PCR analysis of wt, deleted and flox alleles of c-myc from sorted B220+IgM+GFP+ spleen cells. gfp-mb1-fl/fl (c-mycfl/fl;mb1cre/+;rosa26gfp/gfp). Experiment representative of three independent experiments with total of 3 mice for each genotype.
Fig. S2. c-Myc is necessary for B lymphocyte differentiation.  
A, B, and C. Flow cytometry analysis of B lymphocytes from BM of mb1-fl/fl and mb1-fl/+ mice. Single-cell suspensions were prepared, stained and analyzed by flow cytometry (see methods). Cells were defined as (Ly6c-NK1.1-DX5-B220+c-Kit+IgM-) pro-B and (Ly6c-NK1.1-DX5-B220+CD25+IgM-) pre-B cells.  
D. Absolute numbers of B lymphocytes in mb1-fl/fl and control mouse BM (n=7) and spleen (n=3).  
E, F Flow cytometry analysis of B lymphocytes in BM of mx-fl/fl and fl/fl control mice. B cell populations were defined as in A, B and C.  
G. Absolute numbers of B cells in Hardy Fractions of plpC-injected mx-fl/fl mice.  
n=4. Data represent one of ≥3 independent experiments. p values are ***p<0.001, **p<0.01, *p<0.05.
Fig. S3. BM B cell progenitors from gfp-mb1-fl/fl do not generate IgM\(^+\) cells in vitro. BM cells were depleted of IgM\(^+\) with beads and cultured with interleukin-7 for 4 days. Experiment representative of three independent experiments. B220\(^+\)IgM\(^-\) cells were isolated from total BM after depletion of IgM\(^+\) cells using biotinylated anti-IgM antibody (Southern Biotechnologies) and streptavidin Dynabeads (Invitrogen). Purity of cells was >97% after magnetic separation, as confirmed by flow cytometry. Cells were cultured in 24-well plates (2 x 10\(^6\) cells/ml) and supplemented with recombinant murine stem cell factor (rSCF, 10 ng/ml), rFlt3 ligand (10 ng/ml) and recombinant murine IL-7 (10 ng/ml; all from Peprotech). Cells were analyzed by flow cytometry at day 4.
Fig. S4. (A) Gene expression by qPCR of sorted pro-B and pre-B cells (B220⁺IgM⁻) from mb1-f/f and mb1-f/+ control mouse BM (mean ± SD for 3 mutant and 3 control mice); numbers indicate the x-fold change (2-ΔCt). (B) Gene expression of B220⁺IgM⁻GFP⁺ BM cells from pIpC-injected gfp-mx-f/f and control f/f mice. A pool of 3 mice was used for each genotype in two independent experiments. (C and D). Flt3 and c-myc expression by qPCR of purified pro-B (Ly6c-NK1.1-DX5-B220+c-Kit⁺IgM⁻) and (Ly6c-NK1.1-DX5-B220+CD25⁺IgM⁻) pre-B cells. Experiment represents 3 mice of each genotype.
Fig. S5. c-Myc deficient pro- and pre-B lymphocytes do not rearrange the T cell Receptor loci. Sorted B220⁺IgM⁺GFP⁺ BM cells from gfp⁻mb1⁻fl/fl, and gfp⁻mb1⁻fl/+ control mice were cultured in the presence of the OP9-DL-1 cell line. Subsequently, cells were subjected to genomic PCR analysis to detect TCR rearrangements. Thymus and spleen DNA were used as positive controls. Experiment representative of three independent experiments with a total of three mice of each genotype.
**Fig. S6.** Luciferase reporter assays. (A) Genomic locus of *ebf1* and *pGL3-ebf1* reporter constructs. *(B, C, D)* *ebf1β*, *e2a* and *pax-5* genomic loci do not respond to c-Myc. Black squares represent E-boxes. *pGL3-E2A* contains 2 conserved and one non-conserved E-Boxes. Data representative of three independent experiments.
Fig. S7. (A) Flow cytometry analysis of the BM of the mice with the indicated genotype. (B) BM cellularity. n=5 ***p<0.001. (C) CD19 surface expression in B220+IgM+ cells in the BM. Flow cytometry experiments are representative of at least three independent experiments.
Fig. S8. qPCR of transcriptional levels of pax5, ebf1, and c-myc in sorted B220+IgM- from ik-mb1-fl/fl, mb1-fl/+, and ik-mb1-fl/+ mice. 3 mice of each genotype were used.
Table S1. Sequence analysis of VH558 - JH3 distal rearrangements in sorted BM B220⁺ IgM⁺ cells from mbl⁻⁻⁻⁻ and fl/fl control mice. Analysis of 24 sequences from mutant and 20 from control cells of 4 independent mice from each genotype are shown. The V, D and J family and designation are indicated in the table, as well as the % of homology with the corresponding germline segment. All V sequences belong to the VHJ558 subgroup, except for some segments of the VHSM7 family, which is related to the VHJ558 but maps 3' of it. We also analyzed the extension and frequencies of P and N regions, finding no differences between mutant and control. Sequences are normal in terms of VDJ diversity.