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c-Myb Promotes the Survival of CD4⁺CD8⁺ Double-Positive Thymocytes through Upregulation of Bcl-xL

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Mechanisms that regulate the lifespan of CD4⁺CD8⁺ double-positive (DP) thymocytes help shape the peripheral T cell repertoire. However, the molecular mechanisms controlling DP thymocyte survival remain poorly understood. The Myb proto-oncogene encodes a transcription factor required during multiple stages of T cell development. We demonstrate that Myb mRNA expression is upregulated as thymocytes differentiate from the double-negative into the metabolically quiescent, small, preselection DP stage during T cell development. Using a conditional deletion mouse model, we demonstrate that Myb-deficient DP thymocytes undergo premature apoptosis, resulting in a limited Tcra repertoire biased toward 5’ Jα segment usage. Premature apoptosis occurs specifically in the small preselection DP compartment in an αβTCR-independent manner and is a consequence of decreased Bcl-xL expression. Forced Bcl-xL expression is able to rescue survival, and reintroduction of c-Myb restores both Bcl-xL expression and the small preselection DP compartment. We further demonstrate that c-Myb promotes transcription at the Bcl2l1 locus via a genetic pathway that is independent of the expression of T cell-specific factor-1 or RORγt, two transcription factors that induce Bcl-xL expression in T cell development. Thus, Bcl-xL is a novel mediator of c-Myb activity during normal T cell development. The Journal of Immunology, 2010, 184: 2793–2804.

The thymic cortex consists largely of quiescent double-positive (DP) thymocytes undergoing Vα-Jα recombination at the Tcra locus. Production of a functional TCR-α-chain leads to replacement of the pre-TCR complex on DP thymocytes with surface expression of a mature αβTCR complex. Thymocytes that successfully assemble an αβTCR have three possible fates based on their reactivity toward self-peptide–MHCs (1). The vast majority of all DP thymocytes fail to produce an αβTCR with sufficient affinity toward the self-peptide–MHC complex to become positively selected and undergo death by neglect. DP thymocytes that express an αβTCR with high affinity toward the self-peptide–MHC complex are potentially autoreactive and induced to die by negative selection. Less than 5% of all DP thymocytes produce an αβTCR with intermediate affinity toward self-peptide–MHC complexes and receive the vital signal for positive selection. Positively selected DP thymocytes are granted continued survival and undergo commitment to either the CD4⁺ single-positive (SP) or the CD8⁺ SP T cell lineage in an MHC-dependent fashion (2).

T cell maturation relies on the balanced proliferation, survival, and differentiation of thymocytes. During the DP stage, withdrawal from cell cycle coincides with the onset of Vα–Ja rearrangements at the Tcra locus (3–5). The quiescent phenotype at this developmental stage has been attributed to antiproliferative functions of the orphan nuclear receptor RORγt (6). DP thymocytes that fail to receive positive selection signals have a short intrinsic lifespan of 3–4 d before undergoing death by neglect (4, 5). To maximize the chances of assembling a productive αβTCR within this time frame, DP thymocytes are able to undergo multiple rounds of Vα–Ja rearrangements at the Tcra locus, testing further distally 3’ located Ja segments (3, 7–9). This mechanism is known as receptor editing and is terminated by positive selection signals or death by neglect (10–12), ensuring that only thymocytes that express a productive αβTCR will survive. Thus, the survival window of preselection DP thymocytes limits the progression of Tcra rearrangements and thereby influences opportunities for positive selection and the generation of a diverse peripheral T cell repertoire (13). The lifespan of unsignaled DP thymocytes must therefore be precisely regulated to balance mechanisms that enforce death by neglect and those that enable sufficient receptor editing. Hyporesponsiveness to cytokine-mediated survival signaling in preselection DP thymocytes has been reported to be responsible for a gradual decrease in metabolic rate and cell volume and increased sensitivity toward death by neglect (14–16). To counteract premature death by neglect, expression of the critical survival factor Bcl-xL, encoded by the proto-oncogene Bcl2l1, is greatly upregulated in the DP stage of T cell development (17, 18). This efficient upregulation has been attributed to the actions of RORγt and the HMG domain containing transcription factor T cell-specific factor-1 (TCF-1) (19, 20). Bcl-xL–deficient DP thymocytes undergo premature apoptosis and display preferential use of proximal 5’ Ja segments (13, 18).

The Myb proto-oncogene encodes the highly conserved c-Myb transcription factor, which can function to activate or repress transcription in a context-dependent manner (21, 22). The greatest amount of Myb expression is found in progenitor cells of each hematopoietic lineage, and downregulation of Myb expression is associated with differentiation (23–27). Myb⁺/+ mutations are embryonic lethal, and embryos die on day E15 due to severe...
anemia caused by failures of both erythroid and myeloid development (28). Evidence from several models points to the involvement of c-Myb in regulating the balance between survival, proliferation, and differentiation that is required for normal hematopoiesis (29, 30). However, downstream mediators of c-Myb activities during hematopoiesis remain poorly understood.

Large amounts of Myb transcripts have been detected in the thymic cortex (26). We previously used conditional mutagenesis at the Myb locus to demonstrate that c-Myb is crucial at multiple stages of thymocyte differentiation, including transition from the double-negative (DN) to the DP stage, survival of preselecope DP thymocytes and differentiation of CD4 SP thymocytes (31). To gain insight into the prosurvival role of c-Myb in DP thymocytes, the current study uses a Cd4-Cre-expressing mouse strain to achieve efficient deletion at the Myb locus in DP thymocytes. We demonstrate that Myb-deficient DP thymocytes undergo premature apoptosis due to decreased Bcl-xL expression. Premature apoptosis occurs in the small preselection DP compartment due to enhanced dependence on Bcl-xL for survival and restricts the 3′ progression of Jα segment usage during Tcrα recombination. Forced Bcl-xL expression is able to rescue survival, and reintroduction of c-Myb restores both Bcl-xL expression and the small preselection DP compartment. Finally, we demonstrate that c-Myb stimulates Bcl-xL expression at the level of transcription through a genetically distinct pathway independent of RORγt and TCF-1 expression.

Materials and Methods

Mice

Mice were used at 4–6 wk of age. Myb+/− and Myb−/− mice were previously described (31). Cd4-Cre (generous gift of Dr. Christopher B. Wilson, University of Washington, Seattle, WA) and Bcl-2tg (generous gift of Dr. Ellen V. Rothenberg, California Institute of Technology, Pasadena, CA) mice were previously described (32, 33). Tcrα−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Tcrα−/− and Rorγt−/− mice (generous gifts of Dr. Zouming Sun, Beckman Research Institute of the City of Hope, Duarte, CA) were previously described (19, 34). These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Virginia.

Flow cytometry and cell sorting

Single-cell suspensions of thymi from 4–6-wk-old mice were prepared and stained with fluorochrome- or biotin-conjugated Abs as described (31). Abs and reagents were purchased as follows: Caltag (Burlingame, CA): anti–CD4 (clone 5G10); anti–Bcl-xL (clone 54H6), and annexin V (clone 5H10); eBioscience (San Diego, CA): anti–CD90.2 PE (clone 53-2.1); Cell Signaling Technology (Beverly, MA): anti–c-Myb (clone 54H6); Jackson ImmunoResearch (West Grove, PA): F(ab′)2 fragment goat-anti-rabbit-IgG (H+L)-APC. Intracellular staining with anti–Bcl-xL was performed according to manufacturer’s recommendations. Apoptosis detection by flow cytometry was performed using the Annexin V-FITC or PE apoptosis detection kit (BD Biosciences, San Jose, CA) and 7-aminomycin D (7-AAD; Molecular Probes, Carlsbad, CA) following the manufacturer’s recommendations. mRNA expression primers are as follows: Myb forward 5′-AAGGGGACAGCA-3′; Myb reverse 5′-TGCCAGTGTTCTCCAAAA-3′; Mlf1 forward 5′-CGGGAGCTCTGTGAAC-3′; and Mlf1 reverse 5′-ACCTCCAGAACCCCGC-3′. For 1 h at room temperature. After washing the membrane three times in PBS-T, the proteins were detected by ECL (Amersham, Piscataway, NJ). Western blotting

Electronically sorted thymocytes were lysed in 20 mM Tris (pH 7.4), 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, and 1% Triton X-100 (41) containing EDTA-free protease inhibitor mixture (Roche, Indianapolis, IN). Western blotting primary Abs: Millipore (Bedford, MA): anti–c-Myb (clone 1-1); Cell Signaling Technology: anti–Bcl-xL (clone 54H6) and anti–RORγt (clone C6D9); and Rockland (Gilbertsville, PA): anti–Mcl-1 (400-014-394). Anti-RORγt was a generous gift from Dr. Dan R. Littman (New York University School of Medicine, New York, NY) (19).

In vitro survival assays

Freshly isolated total thymocytes were cultured in RPMI 1640 growth medium supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, and 2 mM l-glutamine (Life Technologies, Carlsbad, CA) and 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO) at 2 × 105 cells/ml with no treatment or 40 mM Z-V-AD(OMe)-FMK (ICN Pharmaceuticals, Aurora, OH) for the indicated amount of time in a humidified chamber with an atmosphere of 5% CO2. To monitor the synchronized differentiation of large and small preselection DP thymocytes in vitro, total thymocytes were negatively selected over MACS CD4 Microbeads (Miltenyi Biotec, Auburn, CA). Flow-through was >95% DN and immature single-positive (ISP) thymocytes as determined by flow cytometry and placed in culture for the indicated amount of time.
MA) was performed using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA).

**Chromatin immunoprecipitation**

Protein was crosslinked to chromatin by adding 1% formaldehyde to 10^7 total thymocytes/ml cold PBS for 10 min on ice. The reaction was stopped by adding 125 mM glycine for 5 min with rocking at room temperature. Cells were pelleted and washed once in cold PBS. Cell pellets were resuspended at 10^7 cells/ml cold cytoplasmic lysis buffer (10 mM Tris-HCl [pH 8], 85 mM KCl, 0.5% Nonidet P-40 [NP-40], 1 mM PMSF, and EDTA-free protease inhibitor mixture; Roche) and incubated on ice for 10 min. Nuclei were resuspended at 10^7 cells/ml in ice-cold sonication buffer (10 mM Tris-HCl [pH 8], 0.1 mM EDTA, 1% NP-40, 1 mM PMSF, EDTA-free protease inhibitor mixture) and sonicated using a model W-375 cell disruptor (Ultrasonics, Plainview, NY) to generate chromatin fragments 200–500 bp in size. Debris was cleared by centrifugation, and sonication buffer was supplemented with 5% glycero and 127 mM NaCl. Chromatin aliquots of 500 µl were pre-cleared using salmon sperm DNA/protein-A agarose slurry for 1 h and immunoprecipitated overnight with either 3 µg anti-RNA polymerase II cTD clone 4H8 (Abcam, Cambridge, MA) or mouse IgG2a,κ isotype control (BD Biosciences) with rotation at 4°C. Immune complexes were collected with 100 ml salmon sperm DNA/protein-A agarose slurry for 1 h with rotation at 4°C. Beads were washed 5 min with rotation at 4°C with low-salt buffer (10 mM Tris-HCl [pH 8], 2 mM EDTA, 0.1% SDS, 1% NP-40, 150 mM NaCl), high-salt buffer (10 mM Tris-HCl [pH 8], 2 mM EDTA, 0.1% SDS, 1% NP-40, 500 mM NaCl), and LiCl buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA, 1% deoxycholate, 1% NP-40, 250 mM LiCl) and twice in TE buffer. All wash buffers were supplemented with protease inhibitors and PMSF. Bound complexes were eluted off the beads in 500 µl elution buffer with rotation at room temperature for 30 min. Formaldehyde crosslinking was reversed in the presence of 200 mM NaCl at 65°C overnight. DNA was phenol/chloroform extracted following RNAse A and proteinase K treatment. RNA polymerase II localization at the thymic Bcl11 transcription start site was detected by qRT-PCR. Chromatin immunoprecipitation (ChIP) primer: IF, 5′-TTGGACACCCGATGAAAG-3′; 1R, 5′-CCGGTTGGAACTTTAGT11-3′; 2F, 5′-AITCCTCTGTCGCCTTCTGA-3′; 2R, 5′-CCCCGGGAAAGTCCTTGTTTA-3′.

**Coculture and transduction of thymocytes on OP9-DL1 stromal cells**

Enriched DN thymocytes were allowed to differentiate on OP9-DL1 cells (generous gift from J.C. Zuniga-Pflucker, University of Toronto, Toronto, Ontario, Canada) (43). A total of 10^3 thymocytes were depleted for DP thymocytes using MACS CD4 and CD8 MicroBeads (Miltenyi Biotec) per the manufacturer’s protocol. Negatively selected thymocytes were cultured overnight at 5 × 10^5/ml on 80% confluent OP9-DL1 monolayer in flat-bottom, 24-well culture plates with αMEM (Invitrogen) supplemented with 20% FBS, 100 U/ml penicillin-streptomycin, and 2 mM l-glutamine (Invitrogen) and 5 ng/ml recombinant murine IL-7 (PeproTech, Rocky Hill, NJ). The following day, cocultures were transduced by spinfection at 700 × g for 90 min using retroviral supernatants in the presence of 8 µg/ml polybrein (Sigma-Aldrich). pMIGR1 (gift from Dr. Warren S. Pear, University of Pennsylvania, Philadelphia, PA) was previously described (44). The MIGR1-c-Myb vector was constructed by subcloning an HA-c-Myb cDNA construct into the BamHI/BglII site of MIGR1. The MIGR1-Bcl-xl vector was a kind gift from Dr. Thomas J. Braciale (University of Virginia, Charlottesville, VA). Retroviral supernatants were produced by transient CaPO4 co-transfection of 293T cells with the RetroMax packaging vector pC-L-Eco (Imgenex, San Diego, CA) and the appropriate MIGR1-based expression vector. Seventy-two hours post-transduction, cocultures were harvested for flow cytometry or electronic cell sorting. For the detection of Bcl-xl, protein expression in MIGR1-c-Myb-transduced DP thymocytes, 40 µM Z-VAD (ICN Pharmacia) was added to cocultures 48 h posttransduction and 24 h preharvest.

**Statistics**

Differences between data sets were analyzed with two-tailed Student t test and a confidence level of 99% for the mRNA microarray and 95% for all other experiments.

**Results**

Myb is highly expressed in preselection DP thymocytes

Myb mRNA is abundantly produced by DN and DP thymocytes compared with SP thymocytes and naive CD4 and CD8 T cells (26, 31). To better resolve changes in Myb mRNA expression during αβT cell development, we performed qRT-PCR analysis on mRNA extracted from electronically sorted populations ranging from DN2 thymocytes to mature, naive T cell subsets in the spleen (Fig. 1A). The greatest amount of Myb mRNA was detected in DN2 and DP thymocytes. Interestingly, the amount of Myb mRNA detected decreased in DN3, DN4, and ISP thymocytes compared with DN2 and DP thymocytes, suggesting that Myb mRNA expression transiently decreases in post β-selection thymocytes and then increases ∼4-fold as thymocytes enter the DP subset. Myb mRNA expression is greatly decreased in CD4 and CD8 SP thymocytes and naive CD4 and CD8 T cells compared with DP thymocytes. The DP compartment was further sorted into CD69− CD44− CD25− CD25+ subsets.

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Expression of Myb mRNA during αβT cell development. Myb mRNA expression was analyzed by qRT-PCR and normalized to the expression of Hprt-1 mRNA. The peak of relative Myb mRNA levels in each experiment is indicated as 1. Data are presented as mean ± SEM (n = 2) and are representative of two mice. A, Relative Myb mRNA levels in DN2 (CD4−CD8− c-Kit+CD44+CD25−), DN3 (CD4−CD8− c-Kit−CD44+CD25−), DN4 (CD4−CD8− c-Kit−CD44−CD25−) thymocytes (70), ISP (TCRβ−CD4+CD8−), and thymic and splenic CD4SP (TCRβ−CD4+CD8−) and CD8SP (TCRβ+CD8−CD4+CD8−) populations. B, Relative Myb mRNA in ISP (TCRβ+CD4−CD8+), large preselection DP (FSCloCD69−CD4+CD8+), small preselection DP (FSCloCD69−CD4+CD8+), and postselection DP subsets (FSCmedCD69−CD4+CD8+). C, Relative Myb mRNA expression in Tcrα−/− ISP (CD4+CD8−), large preselection DP (CD4+CD8−FSClo), and small preselection DP (CD4+CD8−FSCmed) subsets.
Myb PROMOTES Bcl-xL EXPRESSION IN THYMOCYTES

Apoptotic cell death in wild-type DP thymocytes occurs most commonly through αβTCR-dependent death by neglect, but also through αβTCR-dependent negative selection. We have previously demonstrated that Lck-Cre mediated deletion in mice homozygous for the loxP targeted allele of Myb (Mybf/f) results in a Tcra-independent survival defect in DP thymocytes and impaired development of CD4SP thymocytes (31). We obtain the same phenotype with Cd4-Cre mediated deletion (Supplemental Fig. 1). These results demonstrate that Myb expression is dynamically regulated during T cell development. The abundance of Myb mRNA expression detected in the small preselection DP subset suggests that a high dosage of c-Myb may be required at this stage during thymocyte development.

c-Myb is required for the survival of small preselection DP thymocytes

Apoptotic cell death in wild-type DP thymocytes occurs most commonly through αβTCR-independent death by neglect, but also through αβTCR-dependent negative selection. We have previously demonstrated that Lck-Cre mediated deletion in mice homozygous for the loxP targeted allele of Myb (Mybf/f) results in a Tcra-independent survival defect in DP thymocytes and impaired development of CD4SP thymocytes (31). We obtain the same phenotype with Cd4-Cre mediated deletion (Supplemental Fig. 2). However, deletion of the Myb allele is more efficient, and we do not detect counterselection for the Myb allele in CD4SP thymocytes as we did with Lck-Cre using Cd4-Cre (31). Interestingly, we did not detect a difference in the number of large preselection DP thymocytes in thymi from Mybf/w/ Tcr–/– mice compared with Mybf/f/ Tcr–/– controls, but we detected a severe reduction in the number of small preselection DP thymocytes (Fig. 2A), suggesting that c-Myb may be particularly important for the survival of the latter subset. To determine if impaired survival of Mybf/f/ Cd4-Cre DP thymocytes is a result of apoptotic cell death, we compared the ability of Mybf/w/ Cd4-Cre and Mybf/f/ Cd4-Cre DP thymocytes to survive in liquid culture in the presence or absence of the pan-caspase inhibitor Z-VAD. After 6 and 24 h in culture, decreased survival was detected in Mybf/f/ Cd4-Cre Tcra–/– DP thymocytes compared with controls (Fig. 2B). Survival of Mybf/f/ Cd4-Cre Tcra–/– DP thymocytes was restored to a level equivalent to controls posttreatment with Z-VAD, suggesting that the decreased number of cells detected in c-Myb–deficient DP thymocytes was due to apoptotic cell death. This result was further confirmed by increased TUNEL staining and caspase-3 activation in cultured DP thymocytes lacking c-Myb (Supplemental Fig. 3). In addition, we observed a preferential depletion of FSC30 cells that lack c-Myb at 24 h postculture, resulting in a higher average FSC in the remaining live Mybf/f/ Cd4-Cre Tcra–/– DP thymocyte pool and an overrepresentation of large preselection DP thymocytes (Fig. 2C). The latter is restored to the level of Mybf/w/ Cd4-Cre Tcra–/– DP thymocytes upon Z-VAD treatment. Consistent with Fig. 2A, this result indicates that the survival defect in Mybf/f/ Cd4-Cre Tcra–/– thymocytes is mainly confined to the small DP compartment. Taken together, these findings demonstrate that decreased survival in c-Myb–deficient DP thymocytes is a consequence of increased apoptotic cell death that occurs in an αβTCR-independent fashion preferentially in the small preselection DP thymocyte subset.

Apoptosis is an important component of normal T lymphopoiesis and can be triggered through the extrinsic or the intrinsic pathway (49). Spontaneous apoptosis of normal DP thymocytes in liquid culture is believed to mimic death by neglect and largely reflect the activity of the intrinsic apoptotic pathway in a cytochrome c/ Apaf-1/caspase-9 apoptosome-independent fashion (50). To better understand how the absence of c-Myb resulted in increased apoptosis of small preselection DP thymocytes, we determined the pathway by which accelerated apoptosis takes place in Myb-deficient preselection DP thymocytes. We cultured Mybf/ Cd4-Cre Tcra–/– and Mybf+/ Cd4-Cre Tcra–/– thymocytes in the presence or absence of the caspase-8–specific inhibitor Z-IETD to examine a possible contribution by the extrinsic apoptotic pathway. Although Z-IETD effectively inhibited apoptosis of anti-CD95–treated C57BL/6 DP thymocytes, it did not prevent premature apoptosis of Mybf/ Cd4-Cre Tcra–/– DP thymocytes in vitro (Supplemental Fig. 4), arguing against a major role for the extrinsic apoptotic pathway in the survival defect caused by the lack of c-Myb. Thus, we turned our attention to members of the Bcl-2 family, which are key effectors of the intrinsic apoptotic pathway. The overexpression or abrogation of several Bcl-2 family members has been associated with dramatic effects on the lifespan of preselection DP thymocytes (17, 18, 32, 35, 51, 52). To determine if forced expression of a prosurvival Bcl-2 family member could rescue survival, Mybf/ Cd4-Cre mice were crossed with Bcl-2tg expressing mice. The Bcl-2 transgene directs expression of human Bcl-2 to the T cell lineage (32) and fully restored cellularity of the small DP compartment (Fig. 2D) as well as in vitro survival of c-Myb–deficient DP thymocytes (Fig. 2E, 2F). These results indicate that c-Myb may counteract the intrinsic apoptotic pathway to promote the survival of small preselection DP thymocytes. Interestingly, Bcl-2tg did not restore but rather enhanced the reduced CD4SP:CD8SP ratio observed in Mybf/ Cd4-Cre thymocytes (Fig. 2D). Previous interpretation of the role for c-Myb in CD4SP lineage development (31, 53, 54) was confounded by the survival defect in c-Myb–deficient preselection DP thymocytes, which limits opportunities for positive selection (13). Our observations in Mybf/ Cd4-Cre thymocytes carrying a Bcl-2tg demonstrate that c-Myb deficiency negatively affects CD4SP lineage representation independent of a survival defect in the small preselection DP compartment.

Decreased lifespan of DP thymocytes in vivo results in the predominant usage of 5′ proximal Tcra Jα segments due to limited 3′ progression of rearrangements along the Jα locus (13). To validate our in vitro survival data, we compared Jα segment usage in DP thymocytes from Mybf/w/ Cd4-Cre and Mybf+/ Cd4-Cre mice, with or without Bcl-2tg expression. cDNA generated from sorted DP thymocytes was subjected to a PCR-based assay (9) in which products amplified by Vα3 and Cα segment-specific primers were sequentially probed for a selection of Jα segments ranging from 5′ (proximal) to 3′ (distal) in location (9, 42). A probe against the Cα segment demonstrates equal loading. Comparison of Jα segment profiles revealed a severe decrease in the usage of the distally located Jα12 and Jα2 gene segments by Mybf/ Cd4-Cre DP thymocytes compared with controls (Fig. 2E). In contrast, both Mybf/w/ Cd4-Cre and Mybf+/ Cd4-Cre DP thymocytes displayed preferential usage of the distal Jα2 segment in the presence of Bcl-2tg expression, consistent with prolonged survival independent of c-Myb. Thus, c-Myb–deficient DP thymocytes do not progress to distal Jα segment usage as a direct consequence of premature apoptosis occurring in vivo.
FIGURE 2. Myb<sup>ff</sup> Cd4-Cre DP thymocytes undergo increased apoptotic cell death in a Tcrα-independent fashion. A. The absolute number of 4–6-wk-old Myb<sup>ff</sup> (fw) Cd4-Cre Tcrα<sup>−/−</sup> and Myb<sup>ff</sup> (ff) Cd4-Cre Tcrα<sup>−/−</sup> thymocyte subsets was calculated based on total thymic cellularity and the percentage of DN (CD4<sup>−</sup>CD8<sup>−</sup>), large preselection DP (CD4<sup>+</sup>CD8<sup>−</sup>FSC<sup>hi</sup>), and small preselection DP (CD4<sup>+</sup>CD8<sup>−</sup>FSC<sup>lo</sup>) thymocytes. Data are presented as mean ± SEM (n = 5; *p = 0.0068, Student t test). B. Assessment of Tcrα<sup>−/−</sup> DP thymocyte survival after 6 and 24 h in culture. Where indicated, thymocytes were cultured in the presence of 40 μM Z-VAD. Cultures were stained for CD4, CD8, 7-AAD, and Annexin 5. Percent survival of DP was defined as the percentage of 7-AAD<sup>−</sup> Annexin 5<sup>−</sup> cells through a CD4<sup>+</sup>CD8<sup>+</sup> gate. n ≥ 5; *p = 0.011; **p = 0.0023. C. Overlayed histograms display the forward scatter distribution of live DP thymocytes of the indicated genotype 24 h postculture in the presence or absence of Z-VAD through a 7-AAD<sup>−</sup> Annexin 5<sup>−</sup> gate. Black and gray numbers above gate indicate the percentage of live Myb<sup>ff</sup> Cd4-Cre Tcrα<sup>−/−</sup> and Myb<sup>ff</sup> Cd4-Cre Tcrα<sup>−/−</sup> DP thymocytes, respectively, within a FSC<sup>hi</sup> gate. D. Absolute number of Bcl-2tg DN (CD4<sup>−</sup>CD8<sup>−</sup>), large DP (FSC<sup>hi</sup>CD4<sup>+</sup>CD8<sup>+</sup>), small DP (FSC<sup>lo</sup>CD4<sup>+</sup>CD8<sup>+</sup>), and CD4SP (CD4<sup>+</sup>CD8<sup>+</sup>) thymocyte populations of 4–6-wk-old Myb<sup>ff</sup> Cd4-Cre and Myb<sup>ff</sup> Cd4-Cre mice. Data are presented as mean ± SEM. n = 3; *p = 0.022; **p = 0.011. E. Assessment of total Bcl-2tg DP thymocyte survival after 24 and 48 h in culture. n = 3; *p = 0.030. F. Histograms display the forward scatter distribution of live DP thymocytes of the indicated genotype 24 h postculture through a 7-AAD<sup>−</sup> Annexin 5<sup>−</sup> gate. Number above gate indicates the percentage of live DP thymocytes within an FSC<sup>hi</sup> gate. G. Decreased 3′ Jα segment use in Myb<sup>ff</sup> Cd4-Cre DP thymocytes due to impaired survival. cDNA was generated from sorted Myb<sup>ff</sup> Cd4-Cre and Myb<sup>ff</sup> Cd4-Cre DP thymocytes in the presence and absence of Bcl-2tg expression. PCR amplification was performed with serially (1/3) diluted cDNA samples using primers specific for Va3 and Ca. PCR products were sequentially probed using oligonucleotide probes specific for the indicated Ja segments as previously described (9, 42). An internal Ca probe was used to normalize input cDNA. Data is representative of two independent experiments.

Decreased Bcl-xL expression in Myb-deficient DP thymocytes

To identify candidate c-Myb target genes that mediate protection from apoptosis through the intrinsic pathway, we performed mRNA expression microarray analysis to compare gene expression profiles in DP thymocytes purified over magnetic beads from four Myb<sup>ff</sup> Cd4-Cre Tcrα<sup>−/−</sup> and four Myb<sup>ff</sup> Cd4-Cre Tcrα<sup>−/−</sup> DP mice. All eight samples were individually hybridized to Mouse Genome 430 2.0 Gene Chips, and the resulting data set was subjected to several selection criteria. We identified genes that displayed a >2-fold differential expression within the gene ontology group regulation of programmed cell death (GO:0043067) (40) (Supplemental Table 1). This analysis revealed a statistically significant 2.4-fold (p < 0.01) decrease in Bcl2l1 transcript levels. In addition, although no proapoptotic members were clearly upregulated, Bcl2l1 was the only antiapoptotic family member significantly downregulated in Myb<sup>ff</sup> Cd4-Cre Tcrα<sup>−/−</sup> DP thymocytes (Fig. 3A). The expression of Bcl2l1, like that of Myb, is upregulated in DP thymocytes (17, 18), making Bcl2l1 an attractive candidate downstream target of c-Myb. In addition to the microarray analysis, we performed qRT-PCR of electronically sorted ISP, large preselection, small preselection, and postselection DP thymocytes from wild-type C57BL/6J mice to better compare the expression of the antiapoptotic Bcl2, McI1, Bcl2a1, and Bcl2l1, and the expression of the proapoptotic Bcl2l1 (Bim) with the pattern of Myb mRNA expression within the DP stage. Out of these five Bcl-2 family members, all capable of influencing DP thymocyte survival (17, 18, 32, 35, 51, 55), Bcl2l1 and McI1 expression (Fig. 3B) both closely parallel Myb (Fig. 1B). However, only Bcl2l1
expression was significantly changed in the absence of c-Myb as identified by the microarray (Fig. 3A), lending further support to a potential biological correlation between c-Myb and Bcl-xL expression.

The decrease in Bcl2l1 mRNA level identified in the microarray experiment was validated in sorted small Mybf/w Cd4-Cre Tcr\(\alpha^{2}/2\) and Mybf/f Cd4-Cre Tcr\(\alpha^{2}/2\) DP thymocytes by qRT-PCR (Fig. 3C). Surprisingly, we did not detect decreased expression of Bcl-xL protein by Western blotting (Fig. 3D), despite undetectable levels of residual c-Myb protein in sorted Mybf/f Cd4-Cre Tcr\(\alpha^{2}/2\) small DP thymocytes. However, because the protein half-life of Bcl-xL greatly exceeds that of c-Myb (56–58), we reasoned that c-Myb–deficient thymocytes that lack expression of Bcl-xL might die rapidly, leaving mainly cells that maintain sufficient expression of Bcl-xL to survive. To address this possibility, Bcl-xL protein expression was examined in Mybf/w Cd4-Cre Tcr\(\alpha^{2}/2\) and Mybf/f Cd4-Cre Tcr\(\alpha^{2}/2\) DP thymocytes that either carried a Bcl-2tg or were treated with Z-V AD in liquid culture. Strikingly, both approaches revealed a marked decrease in Bcl-xL protein in c-Myb–deficient compared with c-Myb–sufficient DP thymocytes (Fig. 3D,3E), suggesting that impaired survival masked the reduction in Bcl-xL protein in freshly isolated preselection DP thymocytes lacking c-Myb. We also compared Mcl-1 protein expression in Mybf/w Cd4-Cre Tcr\(\alpha^{2}/2\) and Mybf/f Cd4-Cre Tcr\(\alpha^{2}/2\) DP thymocytes with or without Bcl-2tg expression. Mcl-1 is another prosurvival member of the Bcl-2 family recently reported to
play a role in the survival of DP thymocytes (55). Consistent with our mRNA expression microarray result, we did not detect a decrease in the amount of Mcl-1 protein in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes with or without Bcl-2tg expression (Fig. 3D). These results demonstrate that the reduced amount of Bcl-xL protein in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes carrying an Bcl-2tg is protein-specific among Bcl-2 family members.

Our observation that Bcl-xL expression is decreased in c-Myb–deficient preselection DP thymocytes (Fig. 3D, 3E) and that the number of small but not large DP thymocytes is reduced in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) compared with Myb\(^{+/+}\) Cd4-Cre Tcr\(^{a−/−}\) littersmates (Fig. 2A) is consistent with two distinct possibilities. First, it is possible that the prosurvival role of Bcl-xL may be selectively required for maintaining the survival of small but not large preselection DP thymocytes. Alternatively, the negative impact of c-Myb deficiency on Bcl-xL expression may be confined to the small preselection DP compartment in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) mice. To distinguish between these two scenarios, we monitored Bcl-xL protein expression and cell survival as Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) and Myb\(^{+/+}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes spontaneously differentiate from the large to the small preselection DP stage in vitro. Adapting a previously described assay (59), thymocytes were depleted of the DP population on magnetic beads and placed in liquid culture, allowing DN and ISP thymocytes that have received pre-TCR signals in vivo to differentiate into the large and small preselection DP stage followed by death by neglect in a synchronous manner. After 20 and 36 h, in vitro-differentiated DP thymocytes from both Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) and Myb\(^{+/+}\) Cd4-Cre Tcr\(^{a−/−}\) mice consisted predominantly of large and small preselection DP thymocytes, respectively (Fig. 4A). At 20 h postculture, reduced intracellular Bcl-xL protein was readily detectable without Z-VAD treatment in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes compared with Myb\(^{+/+}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes (Fig. 4B), whereas no significant difference was detected in their ability to survive (Fig. 4C). This result demonstrates that Bcl-xL protein expression is significantly reduced in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) large DP thymocytes without negatively impacting their survival and is consistent with a model where c-Myb and Bcl-xL are not required for the survival of large preselection DP thymocytes. This is in contrast to 36 h postculture, when we observed no difference in Bcl-xL protein expression (Fig. 4B) but significantly impaired survival in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes (Fig. 4C), indicative of a survival defect in DP thymocytes harboring reduced Bcl-xL protein. This phenotype closely resembles our observations in total thymocyte cultures without Z-VAD treatment (Fig. 3E), likely because total DP thymocytes, much like synchronously differentiating DP thymocytes at 36 h postculture, are predominantly composed of small preselection DP thymocytes. Taken together, these results demonstrate that Bcl-xL deficiency is initiated prior to entering the small preselection DP stage in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) mice and thus support a model where thymocyte survival becomes dependent on Bcl-xL at a point near the end of the proliferative large preselection DP stage or as developing thymocytes enter the quiescent small preselection stage.

Exogenous c-Myb restores the expression of Bcl-xL in c-Myb–deficient preselection DP thymocytes

To confirm that Bcl-xL is a downstream effector of c-Myb–mediated survival in DP thymocytes, we determined if an exogenous source of c-Myb could restore Bcl-xL expression and cell survival in Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes. We used an in vitro system in which thymocytes from Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) and Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) mice were depleted of the DP population on magnetic beads and placed in coculture with OP9-Delta1-like 1 (DL1) stromal cells (43). Thymocytes were subsequently transduced with a c-Myb cDNA-containing retrovirus (MIGR1-c-Myb) and allowed to differentiate into DP thymocytes in coculture. Transduced live DP thymocytes (7-AAD\(^−\), Thy1.2\(^−\), GFP\(^+,\) CD4\(^+,\) CD8\(^−\)) were electronically sorted 72 h postinfection and subsequently either analyzed for Bcl2l1 mRNA expression or cultured for an additional 24 h to assess survival (Fig. 5A). A decreased percentage of MIGR1-transduced Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes was observed on day 4 of coculture compared with MIGR1-transduced control DP thymocytes (Fig. 5B), consistent with impaired survival in preselection DP thymocytes lacking c-Myb. Further, decreased survival upon additional culture of MIGR1 transduced Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes confirmed that the defect is intrinsic to the DP compartment and not a consequence of impaired DN to DP transition (Fig. 5C). By the same criteria, MIGR1-c-Myb–transduced Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) thymocytes displayed complete restoration of survival. Importantly, qRT-PCR revealed restored expression of Bcl2l1 mRNA and protein in sorted MIGR1-c-Myb–transduced Myb\(^{+/−}\) Cd4-Cre Tcr\(^{a−/−}\) DP thymocytes compared with MIGR1-c-Myb–transduced control DP thymocytes (Fig. 5D, 5H), suggesting that...
FIGURE 5. An exogenous source of c-Myb restores Bcl-xL expression and survival in Mybf/f Cd4-Cre Tcrα−/− DP thymocytes. A, Experimental plan to rescue survival of DP thymocytes by retroviral transduction in OP9-DL1 stromal cell cultures. B, Day 4 cocultures transduced with MIGR1-c-Myb or MIGR1 were analyzed by flow cytometry for CD4 and CD8 surface expression through a Thy1.2+7-AAD−GFP+ gate. Results are representative of four separate experiments. C, DP thymocytes transduced with MIGR1-c-Myb or MIGR1 were electronically sorted on day 4 of coculture and cultured for an additional 24 h to assess spontaneous apoptosis as measured by Annexin V and 7-AAD staining. Results are presented as mean ± SEM (n = 2) and are representative of four separate experiments. D, Transduced DP thymocytes from Fig. 4B were electronically sorted, and Bcl2l1 mRNA expression was analyzed by qRT-PCR.
C-Myb regulates Bcl2l1 expression in preselection DP thymocytes. In addition, we repeatedly observed increased accumulation of Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> FSC<sup>lo</sup> DP thymocytes when transduced with MigR1-c-Myb (Fig. 5F). This observation is consistent with a preferential depletion of the small preselection DP compartment observed with cultured Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> thymocytes (Fig. 2C) and a greater requirement for c-Myb- and Bcl-xL-mediated survival by small but not large preselection DP thymocytes. Finally, we demonstrate that MigR1-Bcl-xl-transduced thymocytes also restore Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> DP thymocyte survival (Fig. 5G, 5H). Thus, Bcl-xL acts as a physiological effector of c-Myb function in small preselection DP thymocytes to prolong a critical survival window.

C-Myb controls Bcl2l1 transcription by a genetically distinct pathway that is independent of Tcf-1 and Rorγt expression

To determine if the decrease in Bcl-xl expression in c-Myb-deficient DP thymocytes is a consequence of reduced transcription at the Bcl2l1 locus, we measured RNA polymerase II localization at the previously identified transcriptional start site (primer set 2) (60) by ChIP. Primers that amplify a site ~1 kb upstream of the transcriptional start site (primer set 1) were used as negative control. qRT-PCR of DNA precipitated with anti-RNA polymerase II revealed a 31-fold enrichment of over isotype control at the transcriptional start site in total Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> thymocytes compared with an 8-fold enrichment in Myb<sup>−/−</sup> Cd4-cre Tcr<sup>−/−</sup> thymocytes (Fig. 6A, right). No enrichment was detected in either sample using the negative control primer set 1 (Fig. 6A, left), demonstrating that c-Myb regulates Bcl-xl expression in preselection DP thymocytes by promoting transcription at the Bcl2l1 locus. DNA sequence analysis identified three potential c-Myb binding sites in the Bcl2l1 promoter region that are conserved between humans and mice. However, anti-c-Myb ChIP in Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> thymocytes did not reveal enrichment of c-Myb localization at either of the three potential binding sites compared with Myb<sup>+/+</sup> Cd4-Cre Tcr<sup>−/−</sup> thymocytes (Supplemental Fig. 5). The efficacy of the anti-c-Myb ChIP was verified by a statistically significant enrichment at a previously identified c-Myb binding site within the Cd53 promoter (61) of Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> thymocytes. These results suggest that c-Myb regulates Bcl2l1 expression at the level of transcription, but likely not through direct binding to the three conserved c-Myb binding sites in the Bcl2l1 promoter.

Deficiency of Tcf-1 or Rorγt results in the premature apoptosis of DP thymocytes due to decreased Bcl-xl expression (19, 20). To determine if the absence of c-Myb results in altered expression of Tcf-1 or Rorγt, we compared their mRNA and protein expression in sorted Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> and Myb<sup>+/+</sup> Cd4-Cre Tcr<sup>−/−</sup> DP thymocytes. To reveal potential changes in protein expression that might be masked by impaired survival, we also measured the amount of Tcf-1 and Rorγt expression by qRT-PCR (Fig. 6D), suggesting that Tcf-1 may indirectly stimulate Bcl-xl expression by upregulating Rorγt expression during T cell development. Taken together, our results demonstrate that c-Myb promotes transcription at the Bcl2l1 locus in DP thymocytes via a genetically distinct pathway, independent of Rorγt and Tcf-1 expression (Fig. 7).

Discussion

Apoptosis is an essential process underlying normal lymphocyte development, and dysregulated control of apoptosis can lead to autoimmune disease or immunodeficiency. The intrinsic lifespan of preselection DP thymocytes regulates the composition and diversity of the Tcr repertoire and is therefore critical to normal T cell development (13). Thymocytes enter the DP stage as large proliferating cells, and initiation of Tcr<sub>a</sub> recombination coincides with the transition of large preselection DP thymocytes into a small, metabolically quiescent subset that is prone to death by neglect (10, 15, 62). We previously reported that c-Myb is required for the survival of DP thymocytes in an αβTCR-independent fashion (31). We now demonstrate that Myb expression is upregulated as thymocytes transition into the DP stage. In the absence of c-Myb, premature apoptosis takes place due to Bcl-xl deficiency in the small preselection DP compartment, where Myb expression peaks during normal T cell development. Thymocytes have developed a mechanism to efficiently increase Bcl-xl expression in the preselection DP compartment to promote survival and increase the opportunity for assembling an αβTCR (13, 17, 18). Our observation that c-Myb-deficient DP thymocytes exhibit a Tcr repertoire that is skewed toward the use of 5′-located 5<sup>a</sup> segments demonstrates that c-Myb plays a crucial role in determining the window of time that is available for developing thymocytes to produce a diverse Tcr repertoire.

Spontaneous apoptosis of DP thymocytes in liquid culture mimics death by neglect and is thought to be controlled by the balanced functions of Bcl-xl and the proapoptotic Bcl-2 family analyzed by qRT-PCR. Data is normalized to Hprt-1 mRNA expression. Results are presented as mean ± SEM (n = 2) and are representative of three separate experiments. E. Intragelular staining for Bcl-xl of MigR1-c-Myb or MigR1-transduced Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> and Myb<sup>+/+</sup> Cd4-Cre Tcr<sup>−/−</sup> DP thymocytes to retain Myb<sup>−/−</sup> Cd4-Cre Tcr<sup>−/−</sup> DP thymocytes with reduced Bcl-xl protein, 40 μM Z-VAD was added to the cocultures 48 h prior to intragelular staining on day 5. Results are representative of three separate experiments. F. Forward light scatter profiles of MigR1-c-Myb or MigR1-transduced Myb<sup>−/−</sup> DP thymocytes on day 4 of coculture measured by flow cytometry. Results are representative of three separate experiments. G. Day 4 cocultures transduced with either MigR1-Bcl-xl or MigR1 were analyzed for CD4 and CD8 expression by flow cytometry through a Thy1.2<sup>+</sup>-AAD<sup>−</sup> GFP<sup>+</sup> gate. Results are representative of three separate experiments. H. DP thymocytes transduced with either MigR1-Bcl-xl or MigR1 were sorted on day 4 of coculture and cultured for an additional 24 h to assess spontaneous apoptosis as measured by Annexin 5 and 7-AAD staining. Results are presented as mean ± SEM (n = 2) and are representative of three separate experiments.
FIGURE 6. c-Myb promotes transcription at the Bcl2l1 locus independent of TCF-1 and RORγt expression. A, RNA polymerase II ChIP in Mybf/f Cd4-Cre Tcrα−/− and Mybf/B Cd4-Cre Tcrα−/− total thymocytes. Precipitated genomic DNA was amplified using primer sets specific to either the transcription initiation site (+1) or a negative control site ∼1 kb upstream (∼1000 bp) by qRT-PCR. Results are normalized to IgG control samples and presented as mean ± SEM (n = 3). Data are representative of two independent experiments. B, Rorγt and Tcf11 mRNA levels in sorted Mybf/B Cd4-Cre Tcrα−/− and Mybf/F Cd4-Cre Tcrα−/− FSC−/− DP thymocytes measured by qRT-PCR and normalized to Hprt-1 mRNA expression. Data are presented as mean ± SEM (n = 2) and representative of two separate experiments. C, Western blot of whole cell lysates from sorted Mybf/B Cd4-Cre Tcrα−/− and Mybf/F Cd4-Cre Tcrα−/− small DP thymocytes with and without Bcl-2tg expression probed for TCF-1, RORγt, c-Myb, and β-actin. Results are representative of three separate experiments. D, Western blot of whole cell lysates from Rorγt−/−, C57BL/6J, and Tcf11−/− total thymocytes probed for TCF-1, RORγt, c-Myb, Bcl-xL, and β-actin. Results are representative of two separate experiments.

members Bax, Bak, and Bim in a cytochrome c/Apaf-1/caspase-9 apoptosome-independent fashion (13, 50–52). Our mRNA expression microarray experiments identified a unique decrease in Bcl2l1 mRNA expression among the antia apoptotic Bcl-2 family members but no obvious changes in the expression of pro-apoptotic Bcl-2 family members in c-Myb–deficient thymocytes. This finding, combined with our ability to rescue survival in c-Myb–deficient thymocytes with exogenously supplied Bcl-xL, is consistent with previous work that described a non-redundant role for Bcl-xL in DP thymocyte survival (18, 19). A recent report examined the synergy of Bcl-xL and Mcl-1 in promoting DP thymocyte survival, implying functional redundancy in vivo (55). To clarify this point, we carefully analyzed Mcl-1 expression and detected no difference in the level of Mcl-1 mRNA or protein in c-Myb–deficient DP thymocytes. Thus, our results demonstrate that expression of Bcl-xL alone is sufficient to have a significant impact on preselection DP thymocyte survival.

Few effectors of c-Myb activity have been identified and tested in physiologically relevant models to date. We demonstrate that the efficient upregulation of Bcl-xL expression in preselection DP thymocytes is controlled at least in part at the level of transcription by c-Myb, as RNA polymerase II localization at the Bcl2l1 transcription start site was decreased in c-Myb–deficient thymocytes. Consistent with a direct role for c-Myb in promoting Bcl2l1 transcription, we identified three consensus c-Myb binding sites in the mouse Bcl2l1 promoter (60) that are conserved between mice and humans. However, we were unable to directly localize c-Myb to these sites by ChIP assay. Thus, c-Myb may promote Bcl-xL transcription through an indirect mechanism. Although direct regulators of Bcl2l1 transcription during T cell development have not been identified, the STAT, Rel/NF-κB, and GATA transcription factor families have been reported to directly facilitate Bcl2l1 transcription in immortalized cell lines of hematopoietic origin (63–65). However, none of these transcription factors was identified as aberrantly expressed by our mRNA expression microarray experiment in preselection DP thymocytes that lack c-Myb (data not shown), arguing against their involvement in mediating c-Myb–dependent transcriptional activation at the Bcl2l1 locus. It remains possible that c-Myb may tether to the Bcl2l1 promoter through protein-protein interactions independently of its DNA binding domain or interact with as yet unidentified regulatory regions that control Bcl2l1 transcription. Irrespective of the precise mode of regulation, we demonstrate that c-Myb controls transcription at the Bcl2l1 locus in small preselection DP thymocytes and that Bcl-xL is a downstream effector of c-Myb.

FIGURE 7. A model depicting the regulatory network that controls Bcl-xL expression in preselection DP thymocytes. Bcl-xL is necessary for the survival of small preselection DP thymocytes during αβT cell development. c-Myb, RORγt, and TCF-1 are all required to efficiently up-regulate Bcl-xL expression in DP thymocytes. RORγt also promotes the large to small preselection DP transition. Increased c-Myb expression in small preselection DP thymocytes promotes the expression of Bcl-xL by a genetic pathway independent of RORγt and TCF-1. Expression of RORγt appears to be TCF-1 dependent.
activity during normal T cell development. The implications of this finding may reach beyond normal T cell development to the association of duplication events at the Myb locus in over 8% of human T-ALL and attendant defects in the control of survival, proliferation, and differentiation in these tumors (66, 67).

With this study, we have made clear that c-Myb, RORγt, and TCF-1 are all necessary components in the transcriptional network responsible for the critical upregulation of Bcl-XL expression in the preselection DP compartment. The expression of these three transcription factors is upregulated in DP thymocytes. The induction of both RORγt and TCF-1 expression is known to require signaling through the pre-TCR (36, 68) as well as cessation of IL-7 receptor signaling during T cell development (59). Our finding that RORγt protein expression is diminished in thymocytes that lack TCF-1 supports a model where TCF-1 indirectly promotes Bcl-XL expression by regulating RORγt expression (Fig. 7). Signals that mediate the upregulation of c-Myb during the DP stage remain elusive. However, Western blot analysis demonstrates that c-Myb protein expression does not control nor is itself controlled by RORγt or TCF-1 protein expression. Thus, our data demonstrate that c-Myb is a component of a distinct genetic pathway that promotes transcription at the Bcl2l11 locus in DP thymocytes independent of TCF-1 and RORγt expression.

Our observation that in Myb−/− Cd4-Cre mice, an apparent decrease in Bcl-XL expression among large preselection DP thymocytes precedes a detectable survival defect that is largely confined to small preselection DP thymocytes reflects an increased dependence on Bcl-XL function as thymocytes differentiate from the former into the latter compartment during normal T cell development. This shift from a Bcl-XL–independent to a dependent phenotype cannot be explained by a simultaneous decrease in the expression of the prosurvival Bcl-2, Mcl-1, or Bcl2a1 or a substantial increase in the expression of the antia apoptotic Bcl2l11 as determined by qRT-PCR. Upon withdrawing from the cell cycle and becoming refractory to external growth factors (15, 69), small preselection DP thymocytes enter a state of decreased glucose metabolism and gradual cellular atrophy that is more reliant upon preselection DP thymocytes enter a state of decreased glucose metabolism and gradual cellular atrophy that is more reliant upon

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental Data

Figure S1

Figure S1. The amount of Myb mRNA contained in pre-selection DP thymocytes decreases upon in vitro stimulation. Total Tcrα- thymocytes were stimulated in liquid cell culture with 10 ng/mL PMA and 10 ng/mL A23187. Cells were harvested for flow cytometry and RNA extraction at the indicated time points. Results are representative of two independent experiments. (a) Thymocytes were surface stained for CD4, CD8 and CD69 and analyzed by flow cytometry. Histogram shows expression of the positive selection marker CD69 on DP thymocytes following stimulation. (b) Relative Myb mRNA levels were measured by quantitative real-time PCR and normalized to Hgprt-1 mRNA expression.
**Figure S2.** *Myb*<sup>ff</sup> Cd4-Cre DP thymocytes display impaired survival.  

(a) Deletion efficiency at the floxed *Myb* locus in a *Myb*<sup>ff</sup> Cd4-Cre mouse. Genomic DNA was extracted from electronically sorted DN (CD4<sup>-</sup>CD8<sup>-</sup>), DP (CD4<sup>+</sup>CD8<sup>+</sup>), CD4SP (CD4<sup>+</sup>CD8<sup>-</sup>) and CD8SP (CD8<sup>+</sup>CD4<sup>-</sup>) thymocyte populations and naïve CD4 and CD8 splenic T cells. The presence of the floxed and deleted *Myb* alleles was analyzed by PCR (Bender et al., 2004). (b) *Myb*<sup>fw</sup> (fw) Cd4-Cre and *Myb*<sup>ff</sup> (ff) Cd4-Cre thymocytes were analyzed for surface expression of CD4 and CD8. Numbers next to gates represent the percentage of cells through a live lymphocyte gate. Data is representative of ≥10 mice of each genotype. (c) Bar graph shows mean +/- SEM of the absolute number of total, DN (CD4<sup>-</sup>CD8<sup>-</sup>), ISP (TCR<sup>hi</sup>CD4<sup>-</sup>CD8<sup>-</sup>), DP (CD4<sup>+</sup>CD8<sup>+</sup>), CD8SP (TCR<sup>hi</sup>CD4<sup>-</sup>
CD8$^+$ and CD4SP (TCR$^{hi}$CD4$^+$CD8$^-$) thymocyte populations of 4-6-week-old $Myb^{fw}$ Cd4-Cre and $Myb^{ff}$ Cd4-Cre mice. $n \geq 6$, *$p = 0.0019$, **$p = 0.0011$, ***$p < 0.0001$ (Student’s t-test). (d) Survival assessment of $Myb^{fw}$ Cd4-Cre and $Myb^{ff}$ Cd4-Cre DP thymocytes after 6 and 24 hrs in culture. Where indicated, thymocytes were cultured in the presence of 40 μM of the pan-caspase inhibitor Z-VAD (ICN Pharmaceuticals, Aurora, OH). Cultures were stained for CD4, CD8, 7AAD, and Annexin 5. Percent survival of DP was defined as the percentage of 7AAD$^-$Annexin 5$^-$ cells through a CD4$^+$CD8$^+$ gate. $n \geq 5$, *$p = 0.045$, **$p < 0.0001$.

**Figure S3.** $Myb^{ff}$ Cd4-Cre DP thymocytes undergo accelerated apoptotic cell death as detected by increased caspase 3 activation and DNA fragmentation. Total $Myb^{fw}$ Cd4-Cre and $Myb^{ff}$ Cd4-Cre thymocytes were placed in liquid culture for 4 hrs and harvested for analysis by flow cytometry. (a) Thymocytes were stained for
surface expression of CD4 and CD8 followed by intracellular staining for active caspase 3 using PE Active Caspase-3 Apoptosis Kit (BD Biosciences, San Jose, CA) per manufacturer’s protocol. (b) DNA fragmentation was measured by terminal dUTP labeling, using the In Situ Cell Death Detection Kit, TMR red (Roche, Indianapolis, IN) per manufacturer’s protocol, followed by surface staining for CD4 and CD8.

Figure S4

![Image](image.png)

**Figure S4.** The survival defect in of Myb<sup>ff</sup> Cd4-Cre Tcr<sup>α<sup>−/−</sup></sup> DP thymocytes is not a result of an increase in extrinsic apoptosis. (a) Validation of the efficacy of Z-IETD. Total thymocytes from Tcr<sup>α<sup>−/−</sup></sup> mice were placed in liquid culture for 6 hrs in the presence of 40 μM of the caspase-8 inhibitor Z-IETD (R&D systems, Minneapolis, MN) or Z-VAD (ICN Pharmaceuticals, Aurora, OH). In addition, 2 μg/mL of anti-mouse CD95 (BD Biosciences, San Jose, CA) was added to the culture where indicated. Survival graph shows that Z-IETD and Z-VAD are equivalent in their ability to prevent CD95 receptor-mediated apoptosis in control DP thymocytes. (b) Thymocytes were cultured in the presence of 40 μM of the indicated caspase inhibitor or 2 μl/ml of DMSO for 24 hrs. Z-
VAD but not Z-IETD significantly prevents accelerated apoptosis in Mybf\textsuperscript{fl} Cd4-Cre Tcr\textalpha\textsuperscript{+/-} DP thymocytes. *p = 0.0056, **p = 0.0050 (Student’s t-test).

**Figure S5**

![Graph showing relative signal](image)

**Figure S5.** No enrichment of c-Myb was detectable at three potential c-Myb binding sites of the Bcl2l1 promoter by ChIP. Anti-c-Myb (Epitomics, Burlingame, CA) precipitated DNA from Mybf\textsuperscript{fl} Cd4-Cre Tcr\textalpha\textsuperscript{+/-} and Mybf\textsuperscript{fl} Cd4-Cre Tcr\textalpha\textsuperscript{+/-} total thymocytes was amplified using primers surrounding three (1-3) potential c-Myb binding sites conserved between humans and mice. Primers surrounding a validated c-Myb binding site in the Cd53 promoter was used as positive control (Lang et al., 2005). Primers surrounding arbitrary sites in the Oct2 promoter (McMurry and Krangel, 2000) and a region upstream of the putative c-Myb binding sites (0) in the Bcl2l1 promoter were used as negative controls. Quantitative real-time PCR results are presented as the mean and SEM of relative fw / ff signal from three sets of mice. Bcl2l1 ChIP primer sequences are as follows:

0 F 5’-TTGGACACCGACATCGAAAG-3’, 0 R 5’-CGCGTGGAACGTTTATGGTT-3’,
1 F 5’-TCTCGATGCCAGTCCCTTT-3’, 1 R 5’-GTTTTGCGGCTGGGAAGTAT-3’,
2 F 5'-TCCTTCCAGAGAGGTCTAGG-3', 2 R 5'-TGGAGACCTCGTTTTTCCTGAG-3',
3 F 5'-ATTCCTCTGTCGCTTCTGA-3', 3 R 5'-CCCCGGAAGGTCTTTTGTAT-3').

Table S1

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<td>1420692 at</td>
<td>16184</td>
<td>positive regulation</td>
</tr>
</tbody>
</table>

Table S1. mRNA microarray was performed comparing DP thymocytes from 4 Myb<sup>bw</sup> Cd4-Cre Tcrα<sup>-/-</sup> and 4 Myb<sup>ft</sup> Cd4-Cre Tcrα<sup>-/-</sup> mice. Table shows genes with statistically significant (p<0.01 Student's t-test) >2 fold differential expression within the gene ontology group Regulation of programmed cell death (GO:0043067) (Ashburner et al., 2000). Data was generated using the GeneSifter microarray data analysis system.