c-Myc mediates pre-TCR–induced proliferation but not developmental progression

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Constitutive and cell-autonomous signals emanating from the pre–T-cell receptor (pre-TCR) promote proliferation, survival and differentiation of immature thymocytes. We show here that induction of pre-TCR signaling resulted in rapid elevation of c-Myc protein levels. Cre-mediated thymocyte-specific ablation of c-Myc in $CD25^+CD44^-$ thymocytes reduced proliferation and cell growth at the pre-TCR checkpoint, resulting in thymic hypocellularity and a severe reduction in $CD4^+CD8^+$ thymocytes. In contrast, c-Myc deficiency did not inhibit pre-TCR–mediated differentiation or survival. $Myc^{-/-}$ double-negative (DN) 3 cells progressed to the double-positive (DP) stage and up-regulated TCRαβ surface expression in the absence of cell proliferation, in vivo as well as in vitro. These observations indicate that distinct signals downstream of the pre-TCR are responsible for proliferation versus differentiation, and demonstrate that c-Myc is only required for pre-TCR–induced proliferation but is dispensable for developmental progression from the DN to the DP stage. (Blood. 2006;108:2669-2677)

Introduction

Immature T cells progress through a number of well-defined developmental stages in the thymus. Commitment of bone marrow–derived T-cell progenitors[1-6] to the T-cell lineage requires stimulation of Notch signaling,[2,3,7,8] and results in the up-regulation of CD25 (double-negative [DN] 2 subset). Survival and proliferation of thymocytes at this stage is supported by cytokines such as interleukin-7 (IL-7) and stem cell factor (SCF). The subsequent $CD44^-CD25^+$ (DN3) stage is marked by the rearrangement of the T-cell receptor (TCR) β, γ, and δ loci. Productive TCRβ gene rearrangements and synthesis of TCRβ chains lead to the surface assembly of a functional pre-TCR, comprising the TCRβ as well as the invariant pTα chain and CD3 subunits. Signals emanating from the pre-TCR promote survival and proliferation of immature thymocytes as well as their differentiation to the $CD4^+CD8^+$ double-positive (DP) stage, effectively instructing immature thymocytes to the αβ T-cell lineage.[9]

Despite recent progress in understanding pre-TCR signaling many questions remain unanswered. It is currently known that the pre-TCR is constitutively localized in plasma membrane glycoprotein-enriched microdomains (GEMs) from where it signals in a cell autonomous manner.[10,11] Proximal events of pre-TCR signaling include the phosphorylation of Lck and Zap70. Assembly of the pre-TCR and activation of this pathway is accompanied by a biphasic calcium mobilization, which appears to be regulated by cytoplasmic IP_3 and plasma membrane store-operated calcium channels (SOCs), resulting in nuclear factor (NF) of activated T cells (NFAT) and NFκB activation.[12] These findings, however, are not sufficient to explain the multiplicity of events following the onset of pre-TCR signaling. Recent evidence both from loss- and gain-of-function approaches indicates that several other genes and signaling pathways are involved in the pre-TCR checkpoint. These include kinases such c-Fyn,[13] Csk,[14] and Pim1,[15] and adaptor proteins such as LAT and SLP-76.[16] Several transcription factors were also shown to be essential at this developmental stage, such as Ikaros,[17] E2A,[18] Runx2,[19] and c-Myb.[20,21] Multiple findings indicate the involvement of a number of signaling cascades, including Notch,[22,23] Wnt,[24] and Hedgehog.[25] The interactions between these pathways, however, the signals that mediate their activation and their orchestration with respect to pre-TCR signaling are currently unknown.

The basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor c-Myc has been described to play a role in lymphocyte development. Members of the Myc family (c-Myc, N-Myc, and L-Myc) play an integral role in proliferation, survival, and differentiation of normal and neoplastic cells. Myc binds E-box DNA motifs as a heterodimer with Max, resulting in cell cycle entry[26] and transcriptional activation or suppression of genes.[27-30] c-Myc has been implicated in cell proliferation[31] as well as the control of cell growth.[32-37] Its expression increases rapidly in response to growth factors[38,39] and B-cell receptor (BCR)[40] or TCR ligation.[41] Immature B and T lymphocytes express both c-Myc and N-Myc, while mature cells express only c-Myc. Assessing the requirement for c-Myc in T-cell development was hampered by the embryonic lethality of c-Myc deficient mice prior to the development of lymphocytes.[42] To bypass this problem, Douglas and colleagues[43] generated chimeric animals from $Myc^{-/-}$ embryonic

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stem (ES) cells and *Rag1*$^{-/-}$ blastocysts in which the *Rag1*$^{-/-}$ cells cannot contribute to the lymphoid lineages. In their study, *Myc*$^{-/-}$ progenitors populated embryonic thymi but had reduced proliferation and failed to develop beyond the late DN stages, leading to the suggestion that c-Myc is essential for development through the pre-TCR checkpoint. c-Myc–deficient cells did not populate adult thymi at all, indicating additional defects at earlier stages of hematopoietic development. More recently, c-Myc has indeed been reported to control the self-renewal of hematopoietic stem cells (HSCs),[44] and its conditional ablation in the bone marrow favored self-renewal over differentiation of HSCs in the stem cell niche.[45] The involvement of c-Myc in early hematopoietic development indicates that studying its role at the pre-TCR checkpoint requires conditional animal models that avoid the accumulation of developmental defects resulting from c-Myc deficiency at earlier stages.

Here we report that c-Myc is rapidly up-regulated upon induction of pre-TCR signaling. To characterize the role of c-Myc at the pre-TCR developmental checkpoint we used mice that allow conditional Cre-mediated thymocyte-specific ablation of this protein starting at the DN3 stage.[46] Our studies indicate that c-Myc is required for the proliferation but not the differentiation or survival signals emanating from the pre-TCR.

Materials and methods

Mice

To generate the TetObeta transgenic mice, the cDNA encoding the TCRβ chain from the 2B4 hybridoma was inserted as a *Sal*I-*Cla*I–blunted fragment in the *Xho*I-*Eco*RV sites of the TetOSB polylinker.[47] A 2.9-kb *Xho*I fragment containing the tet operator, minimal cytomegalovirus (CMV) promoter, rat β-globin intron, TCRβ cDNA, and rat β-globin polyadenylation signal was used for the transgenic mice. Primer pairs for genotyping and deletion polymerase chain reactions (PCRs) were as follows: *Lck*Cre transgene 5′-ATCGCTCGACCAGTTTAGT-3′ (forward), 5′-CGATGCAACGAGTGATGA-3′ (reverse). The floxed *Myc* allele was detected with 5′-GCCCCTGAATTGCTAGGAAGACTG-3′ (forward) and 5′-CCGACCGGGTCCGAGTCCCTATT-3′ (reverse). All mice were kept under specific pathogen–free conditions in the animal facilities of Tufts-New England Medical Center according to protocol no. 49-03 approved by the Institutional Animal Care and Use Committee.

Flow cytometry and antibodies

Four-color fluorescence-activated cell-sorter (FACS) staining was performed as described.[48] Antibodies were from BD PharMingen (San Diego, CA): anti-CD3ε–phycoerythrin (PE), –biotin (17A2 and 500A2), anti-B220–CyChrome (RA3.6B2), anti-CD4–fluorescein-5-isothiocyanate (FITC), –CyChrome, –PE, –allophycocyanin (APC), anti-CD8–FITC, –CyChrome, –PE, –APC (53.6.7), anti-TCRβ–PE, –CyChrome (H57), anti-TCRγδ–PE, –biotin (GL3), anti–pan-NK–PE, –biotin (DX5), anti-CD44–FITC, –PE (IM7), anti-CD25–APC (PC61), anti-Gr1–PE, –biotin (RB6.782), anti-CD11b–PE, –biotin (M1/70), and anti-Ter119–PE, –biotin. Biotinylated antibodies were detected with streptavidin-PE, -CyChrome, or -APC. The FITC–Annexin V labeling kit was from BD PharMingen. Intracellular Bcl-2 was analyzed using anti–murine Bcl-2 (monoclonal antibody [mAb] 3F11; BD PharMingen) and purified hamster IgG (antitrinitrophenol; BD PharMingen) as an isotype control. To analyze DN thymocytes, mature cells expressing lineage (lin) markers (CD4, CD8, TCRβ, TCRγδ, CD19, Gr1, Mac1, Ter119, and DX5) were electronically excluded. Intracellular TCRβ staining was performed as published.[49] FACS analysis was performed on a Cyan flow cytometer (DakoCytomation, Fort Collins, CO) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

DN thymocytes were enriched by depletion of lin$^+$ cells using streptavidin-conjugated magnetic beads (Dynal, Oslo, Norway). Cell sorting was performed on a MoFlo cell sorter (DakoCytomation).

Semiquantitative RT-PCR

mRNA was extracted from sorted cells using the High-Pure RNA Isolation Kit (Roche, Indianapolis, IN). cDNA from 50 000 cells was prepared with the Superscript-II RT kit (Invitrogen, Carlsbad, CA). Samples were equilibrated with respect to β-actin using SYBR Green quantitative PCR on an OpticonII machine (Bio-Rad, Hercules, CA). Semiquantitative PCR was performed on 1:5 serial dilutions. All PCR amplifications used touchdown conditions reaction volume was 30 μL. Primer pair sequences were (forward, reverse): p53, 5′-CCCGAGTATCTGGAAGACAG-3′, 5′-ATAGGTCGGCGGTTCAT-3′; Bcl-x_L, 5′-AGCAACCGGGAGCTGGTGGTCGAC-3′, 5′-GACTGAAGAGTGAGCCCAGCAGA-3′; TCRβ, 5′-AGCTGAGCTGGTGGGTGAATGG-3′, 5′-CCTCTGGCCACTTGTCCTCCTCTG-3′; pTα, 5′-GGCACCCCCTTTCCGTCTCT-3′, 5′-TTTGAAGAGGAGCAGGCGCA-3′; c-Myc, 5′-TCACCAACAGGAACTATGAC-3′, 5′-AAGCTCTGGTTCACCATGTC-3′; N-Myc, 5′-GATGATCTGCAAGAACCCAG-3′, 5′-GGATGACCGGATTAGGAGTG-3′; cyclin D2, 5′-CTTCCAAGCTGAAAGAGACC-3′, 5′-TACCCAACACTACCAGTTCC-3′; cyclin D3, 5′-CGAGCCTCCTACTTCCAGTG-3′, 5′-GGACAGGTAGCGATCCAGGT-3′; cyclin E1, 5′-TCCTGGCTGAATGTCTA-3′, 5′-CTTCTCTATGTCGCACCA-3; and β-actin, 5′-TGGAATCCTGTGGCATCCATG-3′, 5′-TAAAACGCAGCTCAGTAACAG-3′.

Quantitative real-time RT-PCR

Quantitative reverse-transcription PCR (qRT-PCR) was performed in real time using an ABI7300 machine (Applied Biosystems, Foster City, CA). $p21^{Cip1}$ and Gadd45α were determined relative to GAPDH expression using TaqMan Gene Expression Assays from Applied Biosystems. c-Myc, Tis21, Id3, and Egr1 were assayed with SYBR Green technology, and expression levels were determined relative to β-actin or as described in "Semiquantitative RT-PCR." Primer sequences were as follows (forward, reverse): β-actin, 5′-ATGGTGGGAATGGGTCAGAA-3′, 5′-TCTC-CATGTCGTCCCAGTTG-3′; Tis21, 5′-ACGCACTGACCGATCATTACA-3′, 5′-GGCTGGCTGAGTCCAATCTGG-3′; Egr1, 5′-TGAGCACCTGACCA-CAGAGTCC-3′, 5′-TGGACGGCACGGCACAGCTCAG-3′; and Id3, 5′-GGCACTGTTTGCTGCTTTAGG-3′, 5′-GTAGCAGTGGTTCATGTCGTC-3′. All qRT-PCR reactions were in triplicate in 20 μL volume containing 0.3 μM of each primer. The conditions for all qRT-PCRs were: 50°C for 2 minutes and 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Western blot

Pellets of total thymocytes were lysed in RIPA buffer supplemented with Protease Inhibitor Cocktail (Roche) and 1 mM PMSF. Samples were resolved on Bis-Tris gradient gels (Invitrogen) and transferred onto nitrocellulose membranes. Secondary antibodies were conjugated to horseradish peroxidase (HRP). The signal was detected using the enhanced chemoluminescence Plus (ECL Plus kit; Amersham Biosciences, Arlington Heights, IL).

CFSE labeling and OP9-DL1 coculture

Sorted cells (10^6-1.5×10^6) were resuspended in 100 μL PBS/ 0.1% BSA and 5 μM CFSE and incubated at 37°C for 10 minutes before washing extensively. Viability after labeling exceeded 60%. CFSE-labeled cells were cultured in 10-cm tissue-culture plates containing a confluent monolayer of OP9-DL1 cells. Cocultures were maintained in the presence of 5 ng/mL Flt3L (PeproTech, Rocky Hill, NJ) and 1 ng/mL IL-7 (R&D Systems, Minneapolis, MN) for 4 to 6 days.

Results

Pre-TCR signaling induces c-Myc expression

We examined the effect of pre-TCR signaling on c-Myc by analyzing a novel mouse strain that allows inducible pre-TCR expression. More specifically, a transgenic strain was generated using a cDNA encoding a TCRβ chain under the control of a

minimal CMV promoter augmented with 7 tet operator sequences (tetO). Inducible TCRβ expression was achieved by crossing onto a second strain[47] that expressed the TetR-VP16 transactivator (tTA) in immature thymocytes under the control of the *Lck* gene proximal promoter (LTH-1). Expression of the transgenic TCRβ chain in the compound mice is suppressed in the presence and induced in the absence of tetracycline. To avoid simultaneous expression of multiple TCRs the inducible pre-TCR mice were crossed onto the *Rag1*$^{-/-}$ background. In the presence of tetracycline the thymic profile of the TetOβ-LTH-*Rag1*$^{-/-}$ mice resembled that of *Rag1*$^{-/-}$ mice with a thymic cellularity of 2×10^6 to 3×10^6, indicating that the drug effectively suppressed TCRβ transgene expression and prohibited pre-TCR assembly. In the absence of tetracycline the tTA induced the expression of the transgenic TCRβ chain leading to the assembly of a functional pre-TCR and developmental progression to the DP stage. Untreated thymi contained 75% to 80% DP cells and had a thymic cellularity of 50×10^6 to 60×10^6 cells. Thymic lobes isolated from newborn mice treated with tetracycline during gestation were placed in organ cultures for 5 days without tetracycline. This resulted in developmental progression with more than 70% of the thymocytes reaching the DP stage and a more than 10-fold increase in the cellularity of the lobes. All DP cells and the majority of DN4 cells expressed intracellular TCRβ chains (Figure 1A), indicating that they had assembled a functional pre-TCR and undergone beta selection. These newly developing thymocytes present an optimal model system for assessing the downstream effects of pre-TCR signaling.

The effect of pre-TCR signaling on c-Myc was examined after induction of TCRβ expression in TetOβ-LTH-*Rag1*$^{-/-}$ thymocytes. To this aim, thymocytes isolated from TetOβ-LTH-*Rag1*$^{-/-}$ or LTH-*Rag1*$^{-/-}$ animals treated with tetracycline were cocultured with OP9-DL1 stromal cells for 24 hours in tetracycline-free culture medium. Suspension cells were recovered from the cocultures and thymocytes (Thy-1$^+$ cells) were sorted. Whole-cell lysates prepared from the sorted cells were used in Western blots to determine the levels of c-Myc protein. After induction of pre-TCR signaling (24 hours), TetOβ-LTH-*Rag1*$^{-/-}$ cells showed an accumulation of c-Myc protein compared with LTH-*Rag1*$^{-/-}$ control cells (Figure 1B), indicating that c-Myc was rapidly activated in response to pre-TCR signals. Similar blots detected no changes in the levels of cyclins D2 and D3 24 hours after induction of pre-TCR signaling (data not shown).

c-Myc expression was further examined after induction of pre-TCR like signaling in Rag-deficient mice by α-CD3ϵ treatment. Injection of the α-CD3ϵ mAb (2C11) in Rag-deficient mice has been previously shown to induce pre-TCR like signaling and promote developmental progression of DN3 cells to the DP stage.[50,51] To determine the effect of these signals on c-Myc expression, lysates were prepared from thymocytes isolated 0, 6, 16, and 48 hours after intraperitoneal injection of purified α-CD3ϵ mAb (50 μg/mouse) in *Rag2*$^{-/-}$ mice and subjected to Western blot analyses. c-Myc protein levels increased gradually throughout the period of observation (48 hours), starting at 6 hours after α-CD3ϵ injection (Figure 1D). To correlate the c-Myc protein levels with the course of the developmental progression induced by α-CD3ϵ treatment, we stained thymocyte suspensions of similarly treated mice with antibodies against CD4 and CD8, or against lineage markers combined with CD44 and CD25, followed by FACS analysis (Figure 1C). After α-CD3ϵ injection (48 hours), thymocytes had not yet developed to the DP stage and were still undergoing the transition from the DN3 to the DN4 stage. Thus, c-Myc induction preceded developmental progression, indicating that it was a consequence of pre-TCR signaling and not a result of the developmental transition.

Taken together, these data suggest that c-Myc expression is induced by pre-TCR signaling and emphasize the need for detailed analyses to determine its role at the pre-TCR–dependent stages of thymocyte development.

Abnormal thymocyte development upon conditional c-Myc ablation in mice

To characterize the role of c-Myc specifically downstream from the pre-TCR, avoiding earlier developmental defects, we used a novel mouse model that allows conditional ablation of c-Myc starting at the DN3 stage of thymocyte development. This was obtained by crossing *Myc*$^{fl/fl}$ mice[46] that carry LoxP sites flanking the coding exons 2 and 3 of the *Myc* gene with mice expressing Cre under the control of the proximal p56^{Lck} promoter[52] (*Lck*Cre).

Cre-mediated deletion of exons 2 and 3 of the *Myc* gene in compound mutant *Lck*Cre-*Myc*$^{fl/fl}$ mice was detectable at the DN3 stage (data not shown), and thus ablation of c-Myc was expected to coincide with the onset of pre-TCR signaling. Efficient and stage-specific Cre-mediated ablation of c-Myc in these mice was

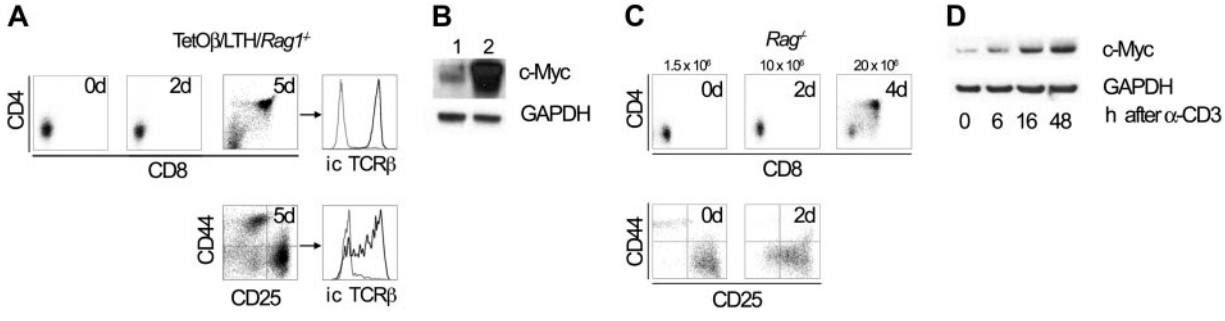

Figure 1. c-Myc expression in response to pre-TCR stimulation. (A) Developmental progression to the DP stage after pre-TCR induction in TetOβ-LTH-*Rag1*$^{-/-}$ thymocytes. FACS analysis of thymocytes from neonatal thymic organ cultures derived from mice that had been treated with tetracycline during gestation. Thymic lobes were kept in organ cultures in the absence of tetracycline for the indicated times. Top dot plots show CD4 versus CD8 and bottom dot plots show CD44 versus CD25 of gated lin$^-$ cells. Histograms show intracellular (ic) TCRβ expression in the DP and the DN4 subsets. (B) c-Myc Western blot of thymocytes from LTH-*Rag1*$^{-/-}$ and TetOβ-LTH-*Rag1*$^{-/-}$ mice that had been treated with tetracycline prior to coculturing with OP9-DL1 cells in tetracycline-free growth medium for 24 hours. Thy1$^+$ cells were sorted from the cocultures and total cell lysates were used for Western blotting. Lane 1 shows LTH-*Rag1*$^{-/-}$; lane 2, TetOβ-LTH-*Rag1*$^{-/-}$. (C) FACS analysis of α-CD3 mAb–induced thymocyte development in *Rag*$^{-/-}$ mice. Dot plots show thymocyte expression profiles for CD4 versus CD8 (top) and CD44 versus CD25 (bottom, data gated on lin$^-$ cells) from *Rag*$^{-/-}$ mice 0, 2, and 4 days after intraperitoneal injection of α-CD3 antibody. (D) c-Myc Western blot from *Rag*$^{-/-}$ mice that were injected with 50 μg of α-CD3 mAb at the indicated time points prior to killing. Total thymic lysates were obtained for Western blotting. Results are representative of 4 independent experiments.

examined by semiquantitative RT-PCR, using cDNA derived from sorted DN3- and DN4-stage thymocytes. Expression of c-Myc was reduced about 5-fold at the DN3 stage and was completely abrogated at the DN4 stage (Figure 2A). Western blot analyses of extracts from similarly sorted cells showed that *Lck*Cre control thymocytes had lower levels of c-Myc protein at the DN3 stage than at the pre-TCR–dependent DN4 stage (Figure 2B). Deletion of the *Myc* gene in *Lck*Cre-*Myc*$^{fl/fl}$ thymocytes severely diminished the expression of c-Myc protein both in the DN3 and DN4 subsets.

To determine the impact of c-Myc ablation on thymocyte development we compared the thymocyte subset distribution (Figure 2C) and thymic cellularity (Figure 2D) of *Lck*Cre-*Myc*$^{fl/fl}$ and control embryos and adult (5- to 8-week-old) mice. Adult *Lck*Cre-*Myc*$^{fl/fl}$ contained approximately 10 times fewer thymocytes ($1.2 \times 10^7 \pm 0.11 \times 10^7$) than *Lck*Cre controls ($8.9 \times 10^7 \pm 0.66 \times 10^7$). This mainly reflected an approximately 30-fold reduction in the number of $CD4^+CD8^+$ DPs in *Lck*Cre-*Myc*$^{fl/fl}$ ($2.7 \times 10^6 \pm 0.17 \times 10^6$) compared with the equivalent *Lck*Cre control cells ($8.4 \times 10^7 \pm 0.92 \times 10^7$) (Figure 2D), and the reduction of the subsequent SP stages. Cellularity at the DN3 stage of *Lck*Cre-*Myc*$^{fl/fl}$ mice was elevated compared with *Lck*Cre controls ($P = .02$; Figure 2D). Interestingly, the number of *Lck*Cre-*Myc*$^{fl/fl}$ DN4-stage thymocytes was comparable with that of *Lck*Cre controls, indicating that development through the pre-TCR checkpoint was not inhibited.

Similar results were obtained from embryonic thymi (Figure 2C-D). Control *Myc*$^{fl/fl}$ embryonic thymi contained on average $4.2 \times 10^6 \pm 1.2 \times 10^6$ cells. This number was reduced by approximately 50% in *Lck*Cre-*Myc*$^{fl/fl}$ mice ($2.3 \times 10^6 \pm 0.6 \times 10^6$), accounting for an approximately 10-fold decrease in the number of DPs ($2.2 \times 10^6 \pm 0.4 \times 10^6$ versus $1.9 \times 10^5 \pm 0.8 \times 10^5$). The DN compartment remained unaltered in profile (Figure 2C) and cellularity (Figure 2D).

Collectively, these data show that conditional ablation of c-Myc at the pre-TCR checkpoint did not affect the transition to the DN4 stage but resulted in the development of fewer DP cells (19% versus 82% adult, and 8% versus 48% embryonic DP cells; Figure 2C). This could either reflect reduced proliferation or increased apoptosis of cells traversing the pre-TCR checkpoint.

c-Myc ablation impairs pre-TCR–dependent proliferation

Following assembly of the pre-TCR, developing thymocytes undergo rapid proliferation at the DN4 stage before progressing to the $CD4^+CD8^+$ DP stage. To address the role of c-Myc in this wave of proliferation, we compared the fraction of cycling cells in *Lck*Cre control and *Lck*Cre-*Myc*$^{fl/fl}$ immature thymocytes. Thymocyte suspensions were stained on the surface to allow identification of the various thymocyte subsets, followed by intracellular staining with the DNA-binding dye 7-amino actinomycin D (7-AAD) and FACS analysis. At the resting DN3 stage, only 4% of *Lck*Cre and 2% of *Lck*Cre-*Myc*$^{fl/fl}$ thymocytes were in the G_2/S/M phases of the cell cycle. The fraction of *Lck*Cre control cells in the G_2/S/M phases at the actively proliferating DN4 stage was 29.1% ± 1.96%, while only 12.2% ± 0.78% of the c-Myc–deficient thymocytes were cycling (Figure 3A), suggesting that c-Myc was required for proliferation at the pre-TCR checkpoint. We also observed that *Lck*Cre-*Myc*$^{fl/fl}$ DN4 stage cells were smaller (Figure 3B) than the equivalent *Lck*Cre cells, probably reflecting the contribution of c-Myc to cell growth.

To ensure that the ablation of c-Myc did not impact pre-TCR assembly we compared the expression of components of the pre-TCR complex in *Lck*Cre control and *Lck*Cre-*Myc*$^{fl/fl}$ thymocytes. Semiquantitative RT-PCR using RNA prepared from sorted DN3- and DN4-stage thymocytes indicated that the expression of pTα and TCRβ mRNA was not impaired in *Lck*Cre-*Myc*$^{fl/fl}$ mice. These cells expressed even higher levels of pTα mRNA compared with the equivalent *Lck*Cre control cells (Figure 3C). Intracellular TCRβ staining revealed that an equal fraction of DN4-stage thymocytes from *Lck*Cre-*Myc*$^{fl/fl}$ and *Lck*Cre mice expressed TCRβ chains, indicating that c-Myc deficiency did not affect the rearrangement and/or the synthesis of TCRβ chains (Figure 3D). These findings indicate that the c-Myc deficiency affects thymocyte proliferation downstream of a properly assembled pre-TCR.

c-Myc deficiency results in deregulation of cell-cycle inhibitors

Although several genes involved in cell-cycle progression and growth control were shown to be transcriptionally regulated by c-Myc,[53,54] the mechanism by which c-Myc mediates cell-cycle progression remains unclear, especially in thymocytes. To trace the impact of c-Myc ablation on genes involved in proliferation, we

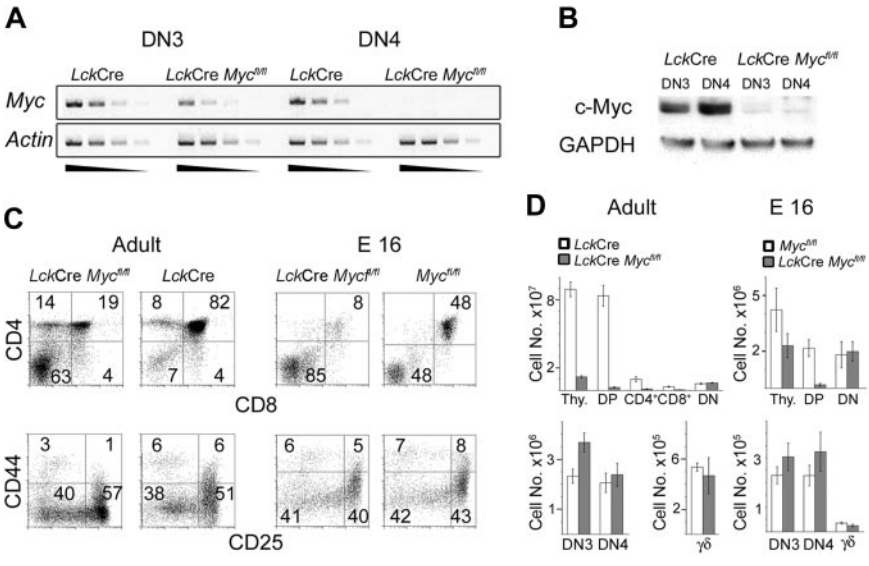

Figure 2. c-Myc ablation at the DN3 stage impacts thymic cellularity and subset distribution. (A-B) Efficiency of c-Myc ablation at the DN3 and DN4 stages of thymocyte development. Semiquantitative c-Myc RT-PCR with 5-fold serial dilutions (A) and c-Myc Western blots (B) were performed on FACS-sorted cells from the indicated mice and subsets. Data shown are representative for 3 independent experiments. (C) FACS analyses for CD4/CD8 (top) and CD44/CD25 (bottom, gated on lin^- events) surface expression in *Lck*Cre-*Myc*$^{fl/fl}$ and *Lck*Cre (Adult = 5-8 weeks old) or *Myc*$^{fl/fl}$ (E 16 = Embryonic day 16) mice. Numbers given indicate the percentage of events in the respective quadrant. Data shown represent observations from more than 10 independent experiments (Adult) and 2 independent experiments (E 16). (D) Cellularity was determined by multiplying the number of total thymocytes with the percentages from panel A (for DN3, DN4 also considering the percentage of lin^- cells). Error bars indicate SD; Thy, total number of thymocytes; and γδ, $TCR\gamma\delta^+$ thymocytes. Numbers of adult animals analyzed to obtain these statistics were as follows ($N_{LckCre\text{-}Mycfl/fl}$, N_{LckCre}): total thymocytes (n = 27, n = 11), DP/$CD4^+$/$CD8^+$/DN (n = 13, n = 6), DN3/DN4 (n = 9, n = 7), and $TCR\gamma\delta^+$ thymocytes (n = 10, n = 5). Embryo data are based on 3 *Myc*$^{fl/fl}$ control embryos and 11 *Lck*Cre-*Myc*$^{fl/fl}$.

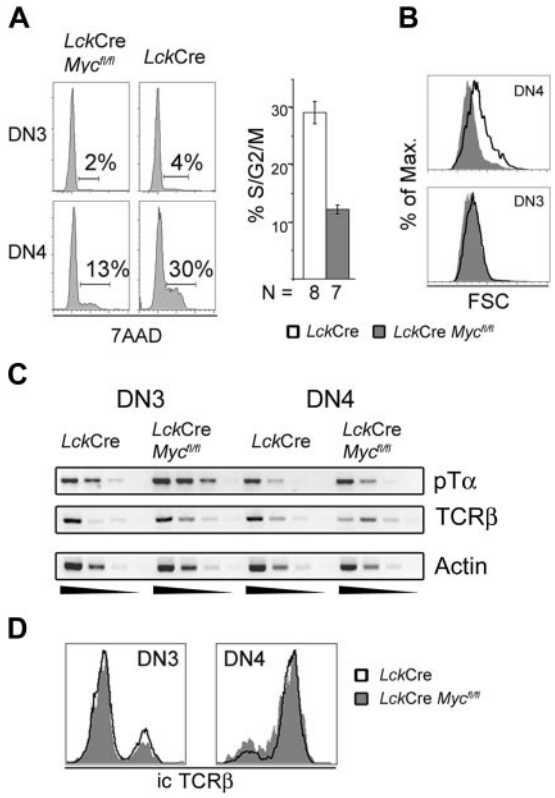

Figure 3. c-Myc ablation inhibits proliferation of DN4-stage thymocytes. (A) 7AAD staining of permeabilized thymocytes. Thymocytes from the indicated mice were surface stained with anti-lin antibodies as well as anti-CD44 and anti-CD25, followed by staining with 7AAD and FACS analysis. Histograms are electronically gated lin$^-$/CD44$^-$/CD25$^+$ (DN3) or lin$^-$/CD44$^-$/CD25$^-$ (DN4) cells. Percentages in histograms represent cells in S/G_2/M phases of the cell cycle. Histogram bars represent cumulative measurements of 8 control and 7 *Lck*Cre-*Myc*$^{fl/fl}$ cycling DN4 cells. Error bars indicate SD. (B) Cell size. Forward scatter (FSC) profiles of the indicated mice and subsets are shown. (C) Semiquantitative RT-PCR for pTα and TCRβ mRNA expression in DN3 and DN4 thymocytes. RT-PCR for β-actin is used as quantity control. Similar results were observed in 3 independent experiments. (D) Intracellular TCRβ expression in DN3 and DN4 thymocytes. Cells were surface stained as in panel A followed by permeabilization and staining with anti-TCRβ antibodies and FACS analysis. Similar results were obtained in more than 5 independent experiments.

compared their expression in *Lck*Cre-*Myc*$^{fl/fl}$ and *Lck*Cre DN3- and DN4-stage thymocytes by semiquantitative and quantitative RT-PCR as well as Western blots (Figure 4A-C). These analyses revealed strikingly elevated protein levels of the cell-cycle inhibitor p27^{Kip} in *Lck*Cre-*Myc*$^{fl/fl}$ DN3 and DN4 thymocytes (Figure 4C). c-Myc–deficient DN4-stage cells also showed elevated expression of the growth arrest and DNA-damage–inducible factor 45 alpha (Gadd45α), and to a lesser extent, of the cell-cycle inhibitor p21^{Cip1} (Figure 4B). Both message and protein levels of the growth-promoting cyclins D2 and D3 appeared unchanged, and the mRNA levels of cyclin E1 were only modestly increased (Figure 4A,C). This is noteworthy considering that cyclin D3 (Figure 4C) has previously been reported to control the proliferative expansion of DN4 and immature single-positive (ISP) thymocytes.[55] Thus, it is likely that the proliferation defect following c-Myc ablation is related to the elevated expression of cell-cycle inhibitors such as p27^{Kip}, p21^{Cip1}, and Gadd45α, which are normally negatively controlled by c-Myc.[56] This notion was further supported by the unchanged expression of genes previously implicated in thymocyte proliferation. These included the inhibitory protein Tis21, described to regulate stage-specific proliferation in fetal thymocytes,[57] the Id3 inhibitor of E2A activity[18] previously shown to be induced after pre-TCR signaling[58] (modestly elevated at the DN4 stage at approximately 70% of control levels; Figure 4B). c-Myc ablation was linked to a probably compensatory transcriptional up-regulation of N-Myc at the DN3 and DN4 stages.

These observations indicate that c-Myc impacts proliferation at the pre-TCR checkpoint by affecting the expression of cell-cycle inhibitors, especially p27^{Kip} and Gadd45α, rather than directly affecting the expression of the cell-cycle–promoting cyclins or other genes reported to promote thymocyte expansion downstream of the pre-TCR.

c-Myc–deficient thymocytes differentiate without proliferating

*Lck*Cre-*Myc*$^{fl/fl}$ thymocytes developed to the CD4$^+$CD8$^+$ DP stage despite reduced proliferation at the DN4 stage, indicating that c-Myc ablation did not influence their differentiation potential. To precisely address the differentiation potential of c-Myc–deficient immature thymocytes we crossed *Lck*Cre-*Myc*$^{fl/fl}$ mice onto the *Rag2*$^{-/-}$ background. These mice showed a complete block at the DN3 stage of thymocyte development. We then induced pre-TCR–like signaling in *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ and control *Lck*Cre-*Rag2*$^{-/-}$ mice by injecting α-CD3ϵ mAb (50 μg/mouse) and analyzed the thymic development 4 days later with respect to

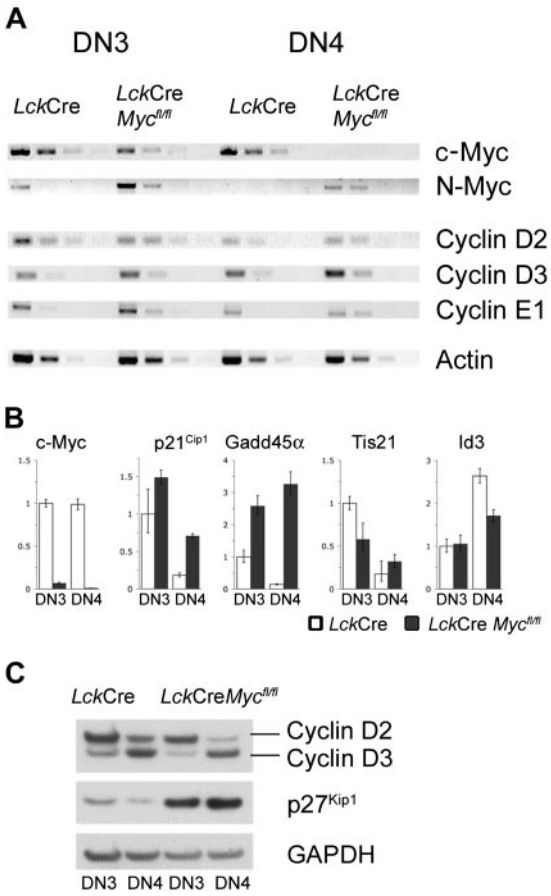

Figure 4. Elevated levels of cell-cycle inhibitors in *Lck*Cre-*Myc*$^{fl/fl}$ thymocytes. (A) Semiquantitative RT-PCR with 5-fold serial dilutions for cell-cycle–related genes performed on c-DNA obtained from FACS-sorted DN3 and DN4 thymocytes. Data sets are representative of observations obtained in 3 independent experiments. (B) Quantitative RT-PCR analyses using RNA prepared from similarly sorted cells were performed in triplicate for the indicated genes. □ represents results for *Lck*Cre control; ■ represents results for *Lck*Cre *Myc*$^{fl/fl}$ mice. Error bars indicate SD. (C) Western blot of sorted cells (2×10^6 per lane) probed with antibodies detecting the indicated proteins. Data are representative of 3 independent experiments.

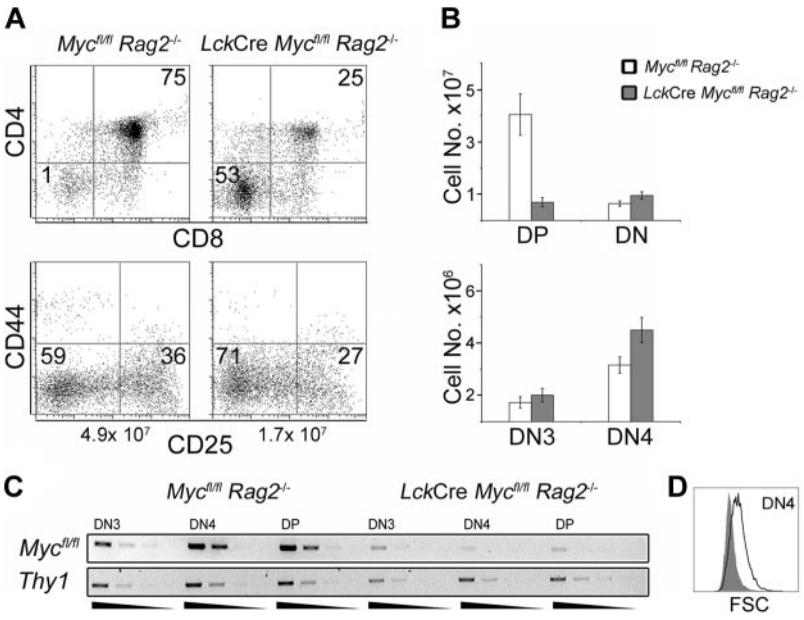

Figure 5. Pre-TCR–like signals induce differentiation of *Lck*-Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ thymocytes. (A) FACS profiles for CD4/CD8 (top panels) and lin$^-$/CD44/CD25 expression 4 days after injection (intraperitoneally) of 50 μg α-CD3 mAb. Numbers given indicate the percentages of cells in the respective quadrants. FACS plots are representative of observations obtained in at least 3 independent experiments. (B) Cellularity was calculated from total thymocyte numbers and the fraction of the indicated subsets. Error bars indicate SD. Eleven *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ and 6 *Lck*Cre-*Rag2*$^{-/-}$ were analyzed to obtain statistics. (C) Semiquantitative PCR with 5-fold serial dilutions was performed to detect the floxed *Myc* allele. Genomic DNA was obtained from FACS-sorted DN3, DN4, and DP cells. (D) Cell size of DN4 stage thymocytes. FSC as observed by FACS.

cellularity and thymocyte subset distribution. Developmental progression was examined by staining for surface expression of CD4, CD8, CD25, and CD44 (Figure 5A). Both *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ and *Lck*Cre-*Rag2*$^{-/-}$ mice progressed developmentally in response to the treatment; however, *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ thymi contained 3- to 5-fold fewer thymocytes than *Lck*Cre-*Rag2*$^{-/-}$ controls. The 2 mouse strains had comparable numbers of DN3- and DN4-stage thymocytes, indicating that c-Myc ablation did not inhibit progression to the DN4 stage. The reduced cellularity of *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ thymi compared with *Lck*Cre-*Rag2*$^{-/-}$ controls was entirely reflected in the reduced fraction (25% versus 75%) and number ($4.0 \times 10^7 \pm 7.9 \times 10^6$ versus $6.9 \times 10^6 \pm 1.73 \times 10^6$; Figure 5B) of DP cells. These data suggested that c-Myc was not required for the developmental transition from the DN to DP stage following pre-TCR signaling.

To rule out the possibility that the progressing cells in α-CD3ϵ–treated *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ mice may have "escaped" timely Cre-mediated deletion of *Myc*, we performed semiquantitative PCR analyses using genomic DNA isolated from sorted DN3, DN4, and DP cells. The floxed *Myc* allele was barely detectable in DN4 and DP stage *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ thymocytes, indicating that these cells had undergone efficient *Myc* deletion (Figure 5C). Moreover, the *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ DN4 thymocytes were smaller than the *Lck*Cre-*Rag2*$^{-/-}$ (Figure 5D), likewise indicating efficient c-Myc ablation. Thus, *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ thymocytes developed to the DP stage despite the lack of c-Myc.

To determine whether developmental progression required cell division we cocultured immature *Lck*Cre-*Myc*$^{fl/fl}$ and *Lck*Cre control thymocytes with OP9-DL1[8] cells. Independently sorted DN3- and DN4-stage thymocytes from *Lck*Cre control and *Lck*Cre-*Myc*$^{fl/fl}$ mice were labeled with CFSE and cocultured with OP9-DL1 cells for 4 days before staining for CD4 and CD8 surface expression and FACS analysis. A substantial fraction of c-Myc–deficient DN3-stage thymocytes up-regulated CD4 (Figure 6A) and CD8 (Figure 6B) surface expression without any cell division, while the developing fraction of *Lck*Cre control DN3 cells had undergone 4 to 5 cell divisions. Likewise, c-Myc–deficient DN4 cells did not divide, but up-regulated CD4 and CD8 surface expression. To determine that these DP cells had undergone proper differentiation we examined their surface expression of TCRαβ (Figure 6C). *Lck*Cre-*Myc*$^{fl/fl}$ and *Lck*Cre control DP cells developing in OP9-DL1 cocultures expressed comparable levels of TCRαβ on their surface. These findings provided both in vivo and in vitro evidence that neither c-Myc

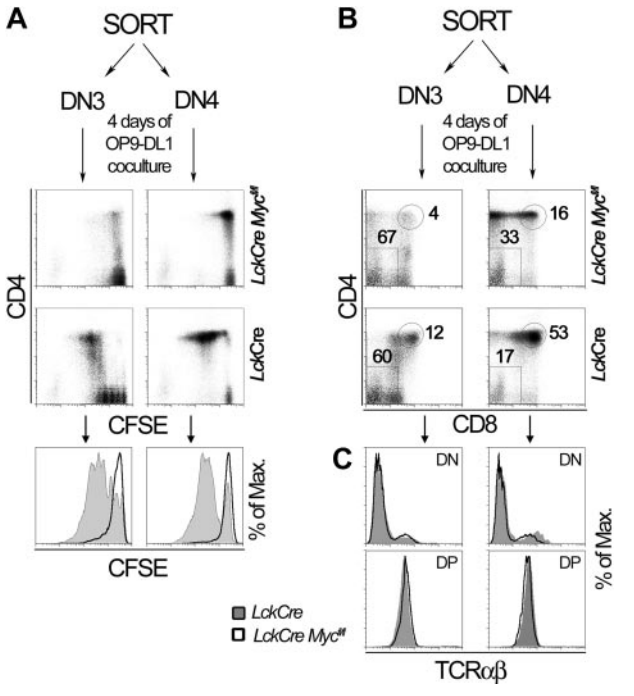

Figure 6. c-Myc–deficient thymocytes differentiate without proliferation. FACS-sorted DN3 and DN4 cells from the indicated mice were labeled with CFSE and cocultured with OP9-DL1 cells for 4 days. Cell suspensions from the OP9-DL1 cocultures were stained with antibodies against CD4, CD8, or TCRαβ and analyzed by flow cytometry. Empty histograms represent results for *Lck*Cre *Myc*$^{fl/fl}$; filled gray histograms represent results for *Lck*Cre control mice. (A) Top panels show 2-parameter dot plots of CD4 versus CFSE staining of the indicated subsets and mice. Bottom panels show histogram overlays comparing CFSE in *LckCre* versus *LckCre-Myc*$^{fl/fl}$ cells after the coculture. (B) Top panels show 2-parameter dot plots of CD4 versus CD8 surface staining of the indicated cells and mice. Numbers represent the percentage of total events in the shown gates. (C) TCRαβ surface expression in the gates shown in the 2-parameter dot plots. Arrows depict the starting populations used in the cocultures. In vitro differentiation was observed in 3 independent experiments.

signaling nor proliferation was required for developmental progression at the pre-TCR checkpoint.

c-Myc ablation does not compromise survival of developing thymocytes

Immature thymocytes that do not receive pre-TCR signals undergo apoptosis. c-Myc has been reported to control cell survival in other experimental systems. To examine whether c-Myc deficiency had an effect on the survival of developing thymocytes, we compared *Lck*Cre-*Myc*$^{fl/fl}$ and *Lck*Cre control thymocytes with respect to their fraction of Annexin V$^+$ cells, as well as the expression levels of the antiapoptotic proteins Bcl-2 and Bcl-x_L and the proapoptotic protein p53. To this aim we stained primary thymocytes with Annexin V as well as antibodies directed against surface markers that allow electronic gating of specific thymocyte subsets (Figure 7A). *Lck*Cre-*Myc*$^{fl/fl}$ and *Lck*Cre control mice had comparable fractions of Annexin V$^+$ cells in the DN3, DN4, and DP subsets, indicating that the c-Myc deficiency did not affect the survival of developing thymocytes. Intracellular staining with antibodies against Bcl-2 revealed that *Lck*Cre-*Myc*$^{fl/fl}$ and *Lck*Cre thymocytes expressed comparable levels of Bcl-2 at the DN3, DN4, and DP stages, further supporting this notion (Figure 7B). We also analyzed the expression levels of Bcl-x_L and p53 mRNA using semiquantitative RT-PCR (Figure 7C) and of p53 protein using Western blot (Figure 7D). The expression level of p53 was unchanged, while Bcl-x_L expression was modestly elevated in the absence of c-Myc.

In summary, these data indicate that c-Myc ablation at the DN3 stage did not impair the pre-TCR–dependent survival signals, and that the reduced thymic cellularity was entirely the result of reduced proliferation.

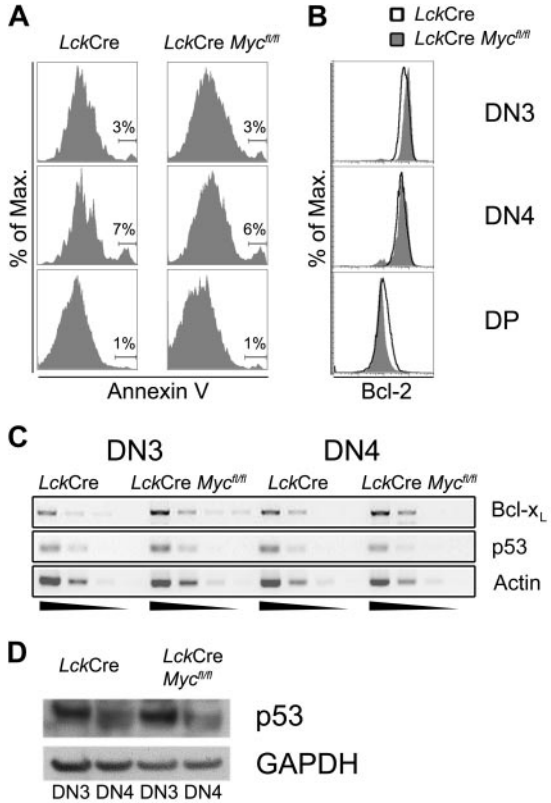

Figure 7. c-Myc ablation does not affect survival of developing thymocytes. (A) Annexin V staining of primary thymocytes. Thymocytes of the indicated mice were stained with antibodies against CD4 and CD8 or against lin, CD44, and CD25 followed by Annexin V and FACS analysis. Histograms of Annexin V staining are electronically gated on the indicated subsets. (B) The same subsets were also analyzed with respect to the expression of intracellular Bcl-2 levels. (C) Expression levels of Bcl-x_L and p53 mRNA in FACS-sorted DN3 and DN4 cells as determined by semiquantitative RT-PCR with 5-fold serial dilutions. Semiquantitative RT-PCR for β-actin was used as quantity control (Actin). Similar results were obtained in 3 independent experiments. (D) Protein levels of p53 in FACS-sorted cells (2×10^6 per lane) as determined by Western blot.

Discussion

Pre-TCR assembly and signaling promotes proliferation, survival, and differentiation of immature thymocytes at the DN3 stage of development, essentially instructing them to the αβT-cell lineage.[9] Using 2 inducible ways to promote this developmental transition we found that an early event following the onset of pre-TCR signaling was the up-regulation of c-Myc. Thus, within hours following induction of pre-TCR signaling, c-Myc protein levels increased, indicating that this molecule was likely involved in the proliferation, survival, or differentiation processes mediated by pre-TCR signaling. We showed that conditional thymocyte-specific ablation of c-Myc impaired cell growth and proliferation of immature thymocytes at the pre-TCR checkpoint. Despite reduced proliferation, the pre-TCR could still signal differentiation and survival to c-Myc deficient thymocytes both in vivo and in vitro. Our findings provide a dissection of pre-TCR signaling, and assign c-Myc specifically downstream of the proliferation but not the differentiation or survival signals.

Three lines of evidence support the suggestion that c-Myc is dispensable for the differentiation signals downstream from the pre-TCR. First, c-Myc–deficient thymocytes progressed efficiently through the DN4 stage, although they yielded a reduced number of DP cells. Second, induction of pre-TCR–like signaling by α-CD3ϵ treatment of *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ mice promoted progression to the DP stage despite the c-Myc deficiency. Third, while control *Lck*Cre DN3-stage thymocytes cocultured with OP9-DL1 cells progressed to the DP stage while undergoing an average of 5 cell divisions, a substantial fraction of *Lck*Cre-*Myc*$^{fl/fl}$ DN3 cells progressed to the DP stage and acquired TCRαβ surface expression with 0 to 1 cell divisions. These data demonstrate that the failure of c-Myc–deficient thymocytes to proliferate does not impact their pre-TCR–dependent differentiation potential. Our observations are in contrast with an earlier report by Douglas and colleagues[43] showing that c-Myc–deficient thymocytes are unable to differentiate to the DP and SP stages in *Rag*$^{-/-}$ *Myc*$^{-/-}$ chimeras. This apparent discrepancy may be related to differences in the experimental systems. While Douglas and colleagues mainly focused on embryonic thymocytes with a constitutive c-Myc deficiency, we conditionally ablated c-Myc immediately prior to the pre-TCR checkpoint, thus avoiding the accumulation of defects from earlier developmental stages.

It is important to distinguish here between the ability of thymocytes to developmentally progress and the number of cells detected in each developmental stage. Since c-Myc–deficient thymocytes progressed to the DP stage without dividing while the equivalent *Lck*Cre cells reached this stage after undergoing 4 to 5 cell divisions, an approximately 32-fold reduction in the number of DP cells would be expected in *Lck*Cre-*Myc*$^{fl/fl}$ mice. This prediction is in line with the reduction in the number of DP cells in *Lck*Cre-*Myc*$^{fl/fl}$ mice. Similarly, fewer *Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ thymocytes treated with α-CD3 are expected to reach the DP stage due to the impairment in proliferation. Indeed, we detected a 5-fold reduction in the number of DP thymocytes in α-CD3ϵ–treated

*Lck*Cre-*Myc*$^{fl/fl}$-*Rag2*$^{-/-}$ mice compared with *Rag2*$^{-/-}$ controls 4 days after injection. Our findings that c-Myc deficiency does not impair differentiation are in line with recent reports showing that in vitro–cultured, c-Myc–deficient HSCs are able to differentiate along the myeloid and lymphoid lineages.[45] Interestingly, in the case of the HSCs—like in thymocytes—this occurs in the absence of significant proliferation. Although our data show that in the absence of c-Myc differentiation is in principle not inhibited in vivo, the reduced numbers of terminally differentiated cells may be devastating and even give the impression of a developmental block.

The c-Myc–deficient DN4-stage thymocytes were cycling at lower frequencies and were smaller, indicating that the proliferative signals attributed to the pre-TCR were impaired in the absence of c-Myc. This proliferative block was detected both in vivo and in vitro but was more dramatic in OP9-DL1 stromal cell cocultures seeded with sorted thymocytes from either the DN3 or the DN4 stage. c-Myc–deficient thymocytes failed to undergo more than 1 cell division in these cultures. The mechanism by which c-Myc promotes thymocyte proliferation may rely on the regulation of cell-cycle inhibitors such as p27^{Kip}, Gadd45α, and p21^{Cip1}.[59-63] This is indicated by the elevated expression of these molecules in c-Myc–deficient thymocytes. Elevated levels of p27^{Kip} have been previously proposed to result in proliferation defects of c-Myc–deficient B cells.[46] Gadd45α has been suggested to determine the susceptibility of a cell to p21^{Cip1}-induced cell-cycle arrest,[64] has been linked to T-cell proliferation,[65] and has also been reported to suppress cell growth via inhibition of the G_2-M–promoting Cdc2 kinase.[66]

c-Myc has been shown to promote cell growth in B cells[33,37] and thymocytes.[34] By contrast, Trumpp and colleagues[67] observed that reduced levels of c-Myc do not affect the size of T cells upon activation. Our finding that c-Myc ablation at the DN3 stage of thymocyte development resulted in small cells that failed to develop into blasts at the DN4 stage suggests that while reduced levels of c-Myc may still be sufficient to mediate cell growth, a complete ablation is not. This explanation is in line with recent findings that c-Myc is likely to regulate cellular growth through ribosome biogenesis,[27,68,69] and that this regulation requires only low levels of c-Myc in the nucleolus.

The reduced thymic cellularity observed in *Lck*Cre-*Myc*$^{fl/fl}$ mice is most likely the result of impaired proliferation, and is not associated with reduced survival. c-Myc has long been thought to sensitize cells to apoptosis, particularly when it is overexpressed. However, conditional ablation of c-Myc was shown not to affect the survival of HSCs[45] or primary B lymphocytes.[70] In line with these observations, we found that spontaneous apoptosis of immature c-Myc–deficient thymocytes was comparable with that of control thymocytes at the equivalent stages. Moreover, we did not detect deregulated expression of several genes implicated in cell survival/death that have been classified as potential c-Myc target genes, such as p53, Bcl-2, and Bcl-x_L.[71-73]

Here we show that c-Myc is rapidly induced upon activation of the pre-TCR. This could be directly controlled by pre-TCR signals, or it could be an indirect consequence. However, the rapid up-regulation of c-Myc protein levels within 6 hours after induction of the pre-TCR argues in favor of a direct control of c-Myc by the pre-TCR. Irrespective of the mechanism by which pre-TCR induces c-Myc, our study reveals for the first time a bifurcation of signaling pathways at the pre-TCR checkpoint and shows that differentiation of thymocytes occurs efficiently in the absence of c-Myc–dependent proliferation.

Acknowledgments

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