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Life and Death Signaling in the Thymus:
Consequences in the Etiology of Cancer and Autoimmunity

By Megan Linn Burger

A dissertation submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy
in
Molecular and Cell Biology
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:
Professor Astar Winoto, Chair
Professor Ellen Robey
Professor Lin He
Professor Danica Chen

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Abstract

Life and Death Signaling in the Thymus:

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Megan Linn Burger

Doctor of Philosophy in Molecular and Cell Biology

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Professor Astar Winoto, Chair

The rigorous process of thymocyte selection is aimed at producing a T cell repertoire capable of responding to a broad range of foreign antigens, but tolerant to self. Only a small percentage of thymocytes entering the selection process emerge from the thymus as mature T cells. Survival and death signals through the T cell receptor (TCR) largely dictate thymocyte fate. Thymocytes with auto-reactive TCRs undergo negative selection and are signaled to die by apoptosis. The Nr4a family of nuclear receptors and the Bcl-2 protein family are critical mediators of thymocyte cell death. Here, we investigate the mechanism by which Nr4a receptors translocate from the nucleus to the mitochondria and induce apoptosis through the mitochondrial death pathway. Through mutagenesis of Nr4a family member Nor-1, we identify regions in the N-terminus of the protein critical for mitochondrial localization. Nr4a proteins have been proposed to induce apoptosis by binding Bcl-2 and converting it to a pro-apoptotic effector through exposure of its BH3 domain. We find that thymocytes expressing a Bcl-2 BH3 mutant transgene exhibit increased resistance to TCR-mediated apoptosis *in vivo*, providing support for Bcl-2 having pro-apoptotic function during negative selection. While death by negative selection is important for inhibiting the production of autoreactive T cells, it remains unclear whether it is necessary for preventing the development of autoimmune disease. Here, we find that Bcl-2 BH3 mutant transgenic mice accumulate activated T cells over time, culminating in the development of multi-organ autoimmunity and early lethality. These data provide new evidence for a crucial role for negative selection in preventing autoimmunity. Aberrant survival signals during thymic selection can lead to the development of T cell malignancies. PTEN, a negative regulator of the PI3-kinase pathway, plays an important role in safeguarding against this fate and in its absence, DP thymocytes form premalignancies that progress to T cell lymphomas. Through the analysis of these premalignancies, we identify two miRNAs that act to suppress malignant transformation by inhibiting proliferation signals downstream of the TCR. Overall, this work provides new insight into the life and death signals important for efficient thymocyte selection and demonstrates how study of their dysregulation in disease can identify novel therapeutic targets.

For my husband, Michael Groesch

I couldn't have done it without you.

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I would like to thank my parents, Danni and Donald Burger, for all their years of love and encouragement. I thank them for supporting me in all my endeavors and allowing me the freedom to pursue anything, even when I thought I wanted to be a professional ballet dancer. I would not have made it this far without the education and many opportunities they provided for me. I thank them for teaching me to work hard and be persistent; qualities that have helped me become a successful scientist.

I am certain I would not have made it through graduate school without the unwavering support of my husband, Michael Groesch. He has been there cheering me on through the good times and bad. Always the sympathetic listener, but dogged optimist, I thank him for never letting me get down on myself. I thank him for all the little things that have added up to so much: bringing me Starbucks so I can keep working, buying me flowers on a bad day, blowing me kisses while I work, hiding while I practice my talks, pouring me a glass of Megan medicine and rescuing me from lab in the middle of the night. Above all, I thank him for all the smiles he's brought to my face over the past 12 years. He brings so much happiness to my life.

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Chapter 1

General Introduction

Apoptosis: a sacrifice for the greater good

Apoptosis, or programmed cell death, is an essential process by which multicellular organisms eliminate unwanted cells during normal development and in the maintenance of tissue homeostasis¹. Dysregulation of this process has been implicated in many human diseases, including autoimmunity, neurodegeneration and malignancy². Apoptotic cell death is primarily orchestrated by a family of aspartate-specific cysteine proteases known as caspases¹. Caspases carry out cleavage of many vital cellular proteins, such as actin and nuclear lamins, and proteolytically activate enzymes that contribute to cellular destruction (e.g. caspase-activated DNase). Caspase activity is initiated downstream of two distinct apoptotic signaling pathways: the extrinsic and intrinsic pathways³.

The extrinsic pathway is initiated by the ligation of death receptors, such as Fas or TNF-R1, resulting in the recruitment of the adaptor protein FADD. FADD recruits procaspase 8 to form the death inducing signaling complex (DISC). The DISC mediates autocatalytic processing of procaspase 8 into its active form, caspase 8, which cleaves and activates the downstream effector caspases 3, 6 and 7 that facilitate cell death⁴. In some cell types, amplification of these death signals through engagement of the intrinsic pathway is necessary to achieve apoptosis. This is mediated by caspase 8 cleavage of pro-apoptotic Bcl-2 family member Bid into its active form, tBid, which acts to inhibit the activity of anti-apoptotic Bcl-2 family proteins³.

The intrinsic, or mitochondrial pathway, is triggered by developmental cues or cytotoxic insults and is under the control of the Bcl-2 protein family, which dictates the integrity of the outer mitochondrial membrane⁵. Apoptosis is initiated by mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release, resulting in the recruitment of apoptotic protease activating factor-1 (APAF-1) and the formation of the apoptosome. The apoptosome facilitates procaspase 9 activation, which subsequently activates downstream effector caspases that ultimately cause death⁵. MOMP is caused by the pore-forming activity of pro-apoptotic Bcl-2 effector proteins such as Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer (Bak) (Figure 1)⁶. Anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bcl-2-related gene long isoform (Bcl-X_L) and myeloid cell leukemia sequence 1 (Mcl-1), bind and inhibit the activity of Bax and Bak, preserving mitochondrial membrane integrity. Pro-apoptotic Bcl-2-homology domain 3 (BH3)-only proteins, including Bcl-2-interacting mediator of cell death (Bim) and p53-upregulated modulator of apoptosis (Puma), sequester anti-apoptotic Bcl-2 proteins, relieving inhibition on Bax and Bak to further promote apoptosis⁶.

Figure 1

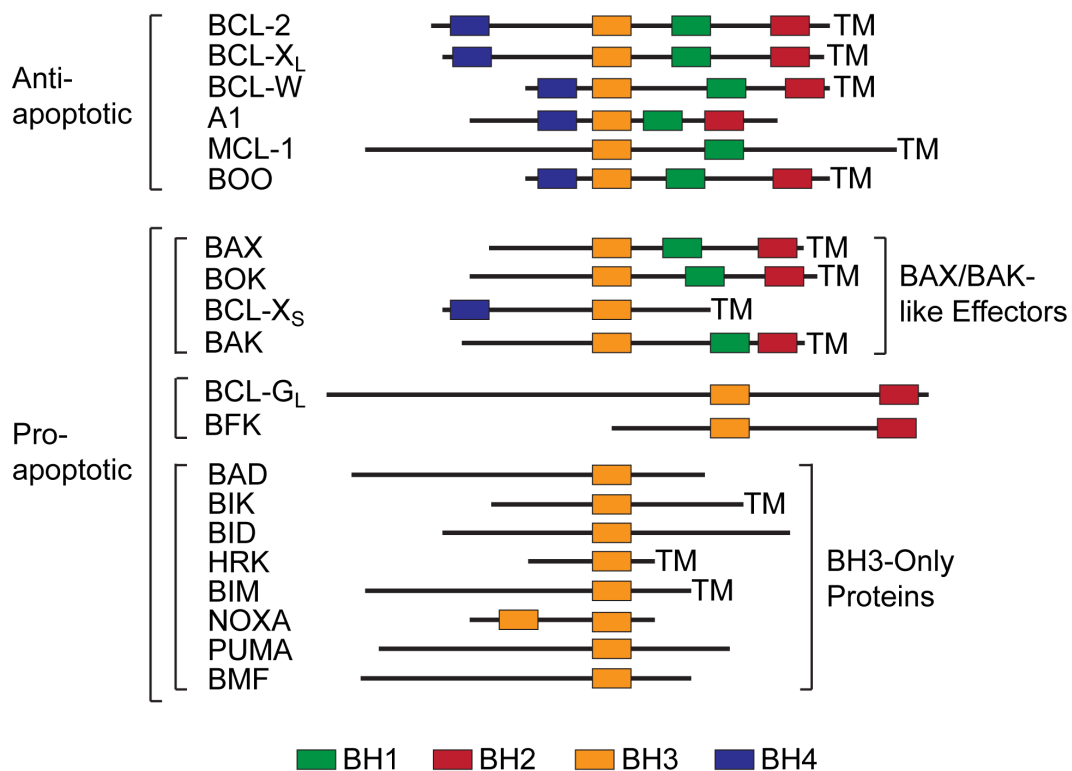


Figure 1. The Bcl-2 protein family. A diagram of the mammalian Bcl-2 family proteins. Anti-apoptotic members contain one of each of the Bcl-2 homology (BH) domains, with the exception of MCL-1. All but BOO have been experimentally shown to be essential for survival of certain cell types. The pro-apoptotic family members can be divided into two groups: the multi-domain BAX/BAK-like effector proteins that facilitate outer mitochondrial membrane permeabilization and the BH3-only proteins that inhibit the activity of the anti-apoptotic Bcl-2 proteins. All BH3-only proteins can bind some of the anti-apoptotic proteins, but BIM and PUMA are unique in their ability to bind all of them⁶. TM = transmembrane domain.

T cell development: a duel with death

Thymocytes, or immature T cells, are derived from bone-marrow precursors and undergo maturation in the thymus. Entering the thymus comes with a high risk of death and less than a 5% chance of successful maturation and exit⁷. Those thymocytes that pass a series of rigorous tests are permitted to survive while all others are fated to die by apoptosis. Only T cells with the functional capacity to recognize antigen through their T cell receptor (TCR), but that demonstrate tolerance to self, are allowed to emerge from the thymus. Hence, apoptosis plays a critical role in the generation of a functional, self-tolerant mature T cell repertoire, an indispensable part of the adaptive immune system.

This highly selective process of T cell development is governed by the architecture of the thymus. The thymus can be divided into two major compartments: the outer cortex and the inner medulla⁸. Thymic seeding progenitors (TSPs) enter the thymus where the cortex and medulla meet, the corticomedullary junction, and gradually lose their multipotency and commit to the T cell lineage through interactions with the thymic stroma⁹. This commitment process occurs during the CD4⁻ CD8⁻ double negative (DN) stage of development, which is separated into four sequential stages, DN1 to DN4, on the basis of cell surface expression of CD44 and CD25 (Figure 2). In early DN stages, Notch1 signaling through interaction with Delta-like 4 (DL4) ligand expressed on stromal cells is key for the repression of alternative gene expression programs, upregulation of T lineage-specific genes, and cell survival⁸.

As thymocytes traverse the DN2 stage, they migrate through the cortex towards the subcapsular zone, a part of the cortex characterized by a high density of cortical thymic epithelial cells (cTECs). During transit, thymocytes begin to rearrange their TCR γ , δ and β loci and continue this process at subcapsular zone as DN3 cells. Successful TCR γ and δ rearrangement specifies the $\gamma\delta$ T cell lineage, while $\alpha\beta$ T cells continue to the β -selection checkpoint⁹. $\alpha\beta$ precursors must test a pre-TCR, composed of a productively rearranged TCR β chain, CD3 chains and an invariant pT α chain, which binds the TCR β chain in the absence of a rearranged α chain. Successful signaling through the pre-TCR is essential for progression past the DN3 stage. Notch1 signaling again participates in promoting cell survival at this stage through activation of the phosphatidylinositol-3-kinase (PI3K)-Akt pathway. Passing the β -selection checkpoint terminates further TCR β rearrangement and initiates a proliferative burst at DN3-DN4⁸. DN4 thymocytes migrate back down into the main cortex and upregulate CD4 and CD8 coreceptor expression to become CD4⁺ CD8⁺ double positive (DP) thymocytes⁹.

At the DP stage, TCR α rearrangement is initiated to assemble a functional $\alpha\beta$ TCR⁸. The fate of these cells is then decided by their TCR affinity for self-peptide presented on major histocompatibility complex (MHC) molecules by cTECs and dendritic cells (DCs). The majority of DP thymocytes (~90%) “die by neglect” because they are unable to engage peptide-MHC (pMHC). These cells undergo a passive form of apoptosis due to the absence of survival signals. DP thymocytes that bind pMHC with low affinity undergo “positive selection” and are signaled to differentiate into CD8 or CD4 single positive (SP) cells based on their TCR affinity for MHC class I or MHC class II molecules, respectively. Strong TCR engagement of pMHC triggers “negative

selection”, resulting in clonal deletion by apoptosis. Negative selection begins in the cortex and continues as positively selected DP thymocytes differentiate into SP cells and migrate to the medulla. Deletion of cells with high reactivity to tissue-restricted antigens is restricted to the medulla where these antigens are expressed and presented by medullary thymic epithelial cells (mTECs) under the control of the autoimmune regulator protein (AIRE)¹⁰. Hence, together, positive and negative selection promote the development of a self-tolerant T cell repertoire that is capable of recognizing a multitude of antigens presented by MHC.

Figure 2

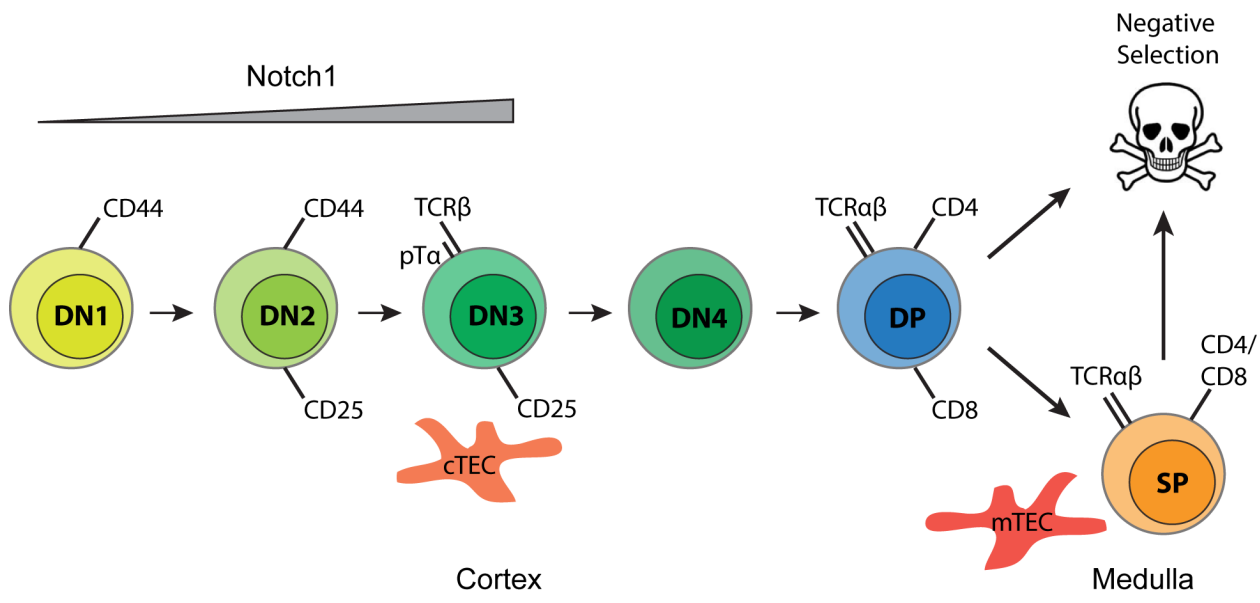


Figure 2. $\alpha\beta$ T cell development. Thymocytes traverse 4 DN stages in the cortex, distinguished by cell surface expression of CD44 and CD25. At DN3, thymocytes undergo TCR β selection, aided by the pseudo-light chain, pT α , and Notch1 survival signaling. β selection is followed by a proliferative burst and differentiation into CD4 CD8 DP thymocytes. At the DP stage, thymocytes test their $\alpha\beta$ TCR for its ability to bind peptide presented by MHC molecules on cTECs or dendritic cells. Signaling through the $\alpha\beta$ TCR promotes positive selection and differentiation into CD4 or CD8 SP thymocytes. DP and SP thymocytes that bind too strongly to peptide-MHC undergo negative selection. mTEC antigen presentation to SP thymocytes in the medulla ensures negative selection of thymocytes reactive to tissue-restricted antigens^{8,10}.

Apoptosis during negative selection: clearing out the bad apples

The Bcl-2 family-regulated, mitochondrial apoptosis pathway is essential for efficient clonal deletion of auto-reactive thymocytes. In particular, the proapoptotic protein Bim is thought to play a crucial role. Bim expression is upregulated in response to negatively selecting TCR stimuli and promotes apoptosis through inhibition of multiple anti-apoptotic Bcl-2 proteins. Bim-deficiency has been shown to substantially rescue thymocytes from deletion in several TCR transgenic models of negative selection. Bim^{-/-} thymocytes are also resistant to Staphylococcus enterotoxin B (SEB) superantigen-mediated deletion. In contrast, the death receptor-mediated apoptosis pathway appears unnecessary for effective clonal deletion. Negative selection occurs normally in Fas (*lpr*), FasL (*gld*) and TRAIL-deficient mice¹¹⁻¹³. Moreover, mice expressing a dominant negative form of FADD, simultaneously blocking signals through multiple death receptors, do not exhibit a defect in negative selection¹⁴.

The Nr4a family of nuclear steroid receptors has also been implicated in promoting apoptosis during negative selection. The family consists of three members, Nur77, Nor-1 and Nurr1. The Nr4a receptors are classified as immediate-early genes and their expression is rapidly and highly induced in response to strong TCR engagement. Recent studies of Nur77-GFP transgenic mice have demonstrated that the level of Nur77 expression directly reflects TCR signal strength¹⁵. T cell-specific over-expression of Nur77 and Nor-1 has been shown to cause massive apoptosis of thymocytes in TCR transgenic models of negative selection^{16,17}. Reciprocally, expression of a dominant negative form of Nur77, that blocks the activity of all family members, significantly inhibited thymocyte apoptosis¹⁶⁻¹⁸. Nur77-deficiency in mice was shown to block negative selection in some TCR transgenic models, but not in others, in line with there being functional redundancy between Nr4a family members^{19,20}. Nr4a triple-knockout mice were found to have a severe defect in T regulatory cell (T_{reg}) generation due to abolished Nr4a-dependent Foxp3 transcription. The quick onset, multi-organ autoimmune disease resulting from loss of T_{reg} immunosuppressive activity masked the effects of Nr4a deletion on thymocyte negative selection²¹. These data suggest that the Nr4a receptors might have a multi-dimensional function during T cell development: inducing thymocyte apoptosis during negative selection and ensuring the generation of T_{reg} cells. It seems logical that Nr4a receptors could function in both capacities, as little distinction exists between these cells fates, with high affinity TCR engagement serving as the primary determinant for both.

While much evidence supports a role for Nr4a receptors in promoting apoptosis during negative selection, their mode of action remains unclear (Figure 3). The Nr4a receptors can act as transcription factors and Nur77's apoptotic capacity has previously been shown to correlate with its transcriptional activity. Transgenic mice expressing a more highly transcriptionally active form of Nur77 exhibit massive thymocyte apoptosis, whereas mice expressing a transcriptionally inactive form of Nur77 do not²². Amongst the Nr4a transcriptional targets that have been identified are two components of the death receptor apoptotic pathway, FasL and TRAIL^{23,24}. However, as discussed above, death receptor signaling appears to be negligible for thymocyte apoptosis during negative selection. Thus, Nur77 might act to promote apoptosis primarily through mechanisms independent of its transcriptional function.

Interestingly, a few recent studies have suggested that the Nr4a receptors might join forces with Bim at the mitochondria to promote thymocyte apoptosis²⁵⁻²⁸. The proposed mechanism was first described for Nur77 in cancer cells. In response to apoptotic stimuli, Nur77 was shown to translocate to the mitochondria and bind Bcl-2, causing Bcl-2 to undergo a conformational change, exposing its BH3 domain. Exposure of the BH3 domain, the “killer” domain of the Bcl-2 protein family, converts Bcl-2 from anti-apoptotic to pro-apoptotic and induces apoptosis²⁹. BH3-exposed Bcl-2 is thought to act in a manner similar to Bim, binding and inhibiting anti-apoptotic Bcl-2 family proteins, though only inhibition of Bcl-X_L has been demonstrated to date³⁰. In thymocytes, Nur77 and Nor-1 were similarly shown to translocate to the mitochondria and induce Bcl-2 BH3 domain exposure in response to strong TCR stimuli²⁶. Inhibition of Nur77/Nor-1 nuclear export inhibited BH3 exposure and markedly protected thymocytes from TCR-mediated apoptosis *in vitro*. Additionally, Bcl-2 BH3 exposure could be detected in TCR transgenic thymocytes undergoing negative selection *in vivo*²⁶. However, the *in vivo* pro-apoptotic function of Bcl-2’s BH3 domain has yet to be unequivocally demonstrated. In cancer cells, injection of a Nur77 cell penetrating peptide (capable of converting Bcl-2) into breast cancer xenografts was able to promote cancer cell apoptosis. However, recent reports of this peptide having cytotoxicity issues has cast doubt on these data (K. Banta unpublished,³¹).

In Chapter 2 of this work, the *in vivo* pro-apoptotic function of the Bcl-2 BH3 domain in thymocyte apoptosis is assessed by creation and characterization of T cell-specific Bcl-2 BH3 domain mutant transgenic mice. Our findings lend further support to Bcl-2 having pro-apoptotic function in thymocytes undergoing negative selection. In Chapter 3, we explore the mechanism regulating Nur77/Nor-1 translocation to the mitochondria. Prior observations in the lab indicate that translocation is dependent on Protein Kinase C (PKC) and calcium signaling downstream of the TCR, however the downstream regulation is unclear²⁵. Our results point to the N-terminus of Nur77/Nor-1 as being critical for promoting nuclear export/mitochondrial localization. These observations will guide future studies to delineate upstream regulation and to directly examine the relative contributions of Nur77/Nor-1 nuclear versus mitochondrial killing functions during thymocyte negative selection.

Figure 3

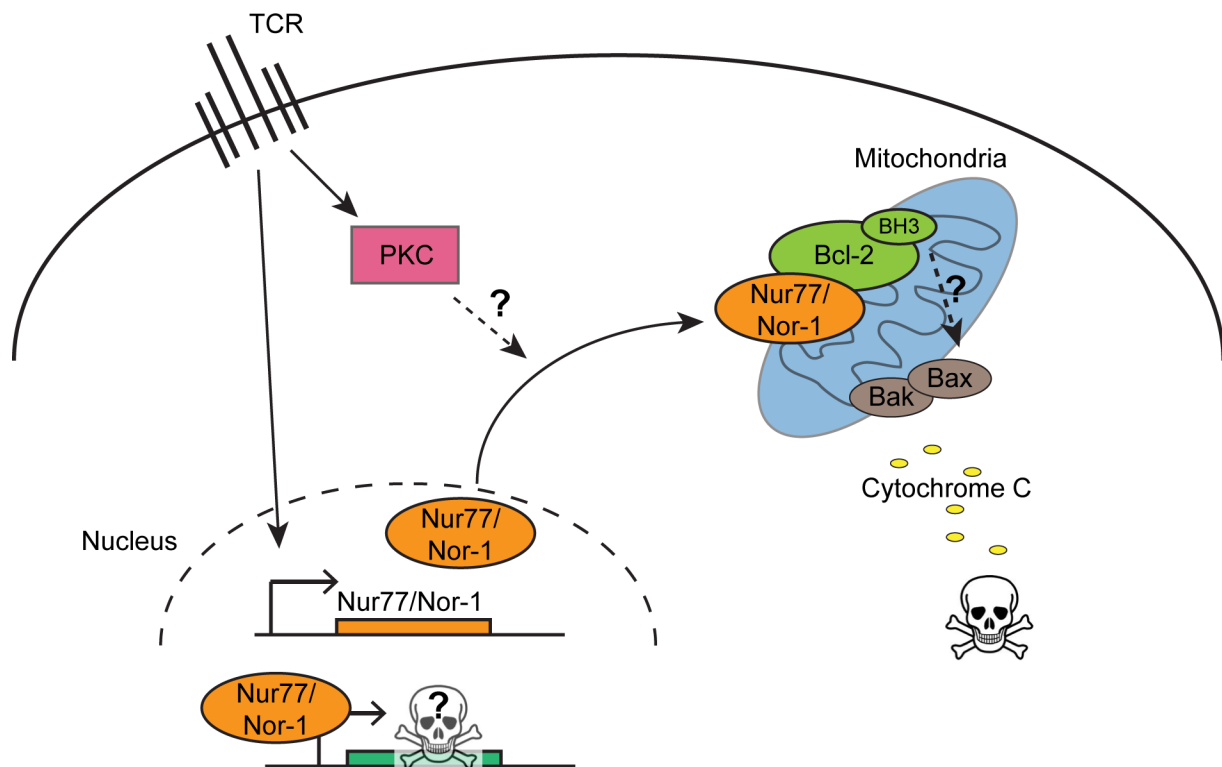


Figure 3. Putative Nur77/Nor-1 apoptotic mechanisms during thymocyte negative selection. In response to strong TCR signals, Nur77/Nor-1 expression is highly upregulated. Nur77/Nor-1 act as transcription factors to promote expression of target genes including FasL and TRAIL, which might play a role in inducing apoptosis of thymocytes through the death receptor pathway. Nur77/Nor-1 also translocate from the nucleus to the mitochondria in a PKC-dependent manner, however regulation downstream of PKC remains unclear. At the mitochondria, Nur77/Nor-1 can bind Bcl-2 and induce the exposure of Bcl-2's BH3 domain. Bcl-2 BH3 domain may act to promote apoptosis at the mitochondria through ill-defined mechanisms^{25,26,32}.

Thymocytes defying death: autoimmunity

Establishing T cell self-tolerance is critical for preventing the development of autoimmune diseases. Elimination of self-reactive thymocytes by negative selection, or establishment of “central tolerance”, serves as the first line of defense. Diversion of some thymocytes with high affinity TCRs to harmless alternative cell fates, such as T_{reg} or CD8 $\alpha\alpha$ cells, also contributes to setting up this barrier³³. However, whether a breakdown in central tolerance is sufficient to cause autoimmune disease remains under debate. This process is also often called “recessive tolerance”, as evidence has been piling up to suggest that “dominant tolerance” mechanisms can generally compensate for a breakdown in central tolerance. Dominant tolerance is established by disabling autoreactive T cells in the periphery through T_{reg}-mediated suppression, peripheral deletion or induction of anergy³⁴. The importance of dominant tolerance for the prevention of autoimmune disease is very clear from the phenotype of Foxp3-deficient mice, which rapidly develop a severe, multi-organ autoimmune disease due to the absence of T_{reg} cells³⁵.

A strong piece of evidence supporting the importance of central tolerance in the prevention of autoimmunity is the phenotype of AIRE-deficient mice. AIRE^{-/-} mice exhibit defective negative selection against tissue-restricted antigens and consequently develop a T cell-mediated, multi-organ autoimmune disease³⁶. However, impaired expression of AIRE in extra-thymic AIRE expressing cells also appears to cause defects in peripheral tolerization of CD4 T cells³⁷. Thus, the relative contribution of the thymic versus peripheral defects remains unclear. Deficiency in Bim also results in a marked defect in negative selection in the thymus, however T cells escaping deletion were determined to be functionally inert³⁸. Nevertheless, Bim^{-/-} mice go on to develop a systemic lupus erythematosus (SLE)-like autoimmune disease, though this disease could be mediated by deletion of Bim in non-T cells³⁹. The disease was also largely ameliorated upon crossing these mice onto the autoimmune resistant, C57BL/6 background^{40,41}. In addition, T cell-specific over-expression of Bcl-2, which can inhibit the activity of multiple pro-apoptotic Bcl-2 family proteins, does not result in autoimmune disease^{42,43}. It is possible, however, that Bcl-2 was not expressed at a high enough level or as discussed earlier, Bcl-2 might be converted into a pro-apoptotic effector by Nur77/Nor-1. Recently, Bim and Puma double-knockout mice were found to develop a multi-organ autoimmune disease similar to that observed in AIRE-deficient mice⁴⁴. These mice had a more pronounced defect in clonal deletion compared to Bim^{-/-} mice and their T cells were able to transfer the disease, suggesting the disease was T cell-derived⁴⁴. While these data provide stronger evidence, a role for non-T cells cannot be ruled out due to germline deletion of Bim and Puma.

In Chapter 2, we unearth new evidence in support of a critical role for central tolerance in preventing autoimmunity. We find that over-expression of a Bcl-2 BH3 mutant transgene specifically in T cells results in a greater defect in thymocyte negative selection compared to over-expression of wild-type Bcl-2. These mice go on to develop multi-organ autoimmune disease, similar to that observed in AIRE^{-/-} and Bim^{-/-}/Puma^{-/-} mice. This study, co-authored by Kenneth K. Leung, Margaux J. Bennett and Astar Winoto, was submitted for publication in May 2014.

Thymocytes cheating the system: cancer

While survival signals are crucial for promoting T cell maturation, especially at the DN stages, aberrant signaling through prosurvival pathways can lead to malignant transformation of thymocytes. For instance, the Notch1 signaling pathway, crucial for early T cell development, also plays a prominent role in driving the development T cell acute lymphoblastic leukemia/lymphoma (T-ALL) from T cell progenitors. Gain-of-function Notch1 mutations have been identified in up to 55% of human T-ALLs⁴⁵. During T cell development, Notch1 expression is critical for passing the TCR β -selection checkpoint and driving the proliferative burst between DN3 and DN4. Notch1 is then abruptly downregulated and remains low in DN4 and DP thymocytes⁴⁶. As many T-ALLs are derived from progenitors having passed the β checkpoint, transformation likely results from defective downregulation of Notch1 during the selection stages. Expression of c-myc, a potent oncogene and a primary Notch target, closely follows the pattern of Notch1 expression in the late DN and DP stages. Hyperactivation of c-myc downstream of constitutive Notch1 signaling is thought to play a major role in promoting excessive cell growth and transformation. Notch1 activation also results in amplification of PI3K signaling by multiple mechanisms, including downregulation of the PI3K negative regulator, Phosphatase and Tensin Homolog (PTEN)⁴⁵.

Aside from Notch1, mutations resulting in hyperactivation of the PI3K pathway are most common in T-ALL, found in up to 48% of cases⁴⁷. 27% of cases have mutations resulting in the inactivation of PTEN⁴⁷. Like Notch1 signaling, PI3K-Akt signaling is required for passage through the DN3 β checkpoint, but aberrant signaling past this stage can cause problems⁴⁸. PTEN-deficient DP thymocytes exhibit increased resistance to apoptosis, allowing them to evade negative selection⁴⁹. Even at the young age of 6-8 weeks, T cell-specific PTEN-deficient (tPTEN^{-/-}) mice begin to show signs of autoimmunity, however the contribution of impaired negative selection to this phenotype remains a subject of debate^{49,50}. PTEN-deficiency also results in hyperactivation of the PI3K-Akt signaling pathway in DP thymocytes and the eventual development of T cell lymphomas⁵¹. Similar to Notch activation, PTEN-deficiency leads to aberrant c-myc activation in DP thymocytes, critical for their transformation⁵⁰. Interestingly, DP thymocytes pause in a state of premalignancy before progressing to full blown CD4⁺ T cell lymphomas⁵¹. In Chapter 4 of this work, we investigate whether dissection of such premalignancies can be used as strategy to identify factors involved in suppressing malignant transformation. Our study identifies two miRNAs able to delay DP cell transformation by negatively regulating TCR signaling and the activation of c-myc. These findings suggest that the study of premalignancies might be a useful way to identify novel tumor suppressive mechanisms and potential therapeutic targets. This work, co-authored by Ling Xue, Yuefang Sun, Chulho Kang and Astar Winoto, was published in *Blood*, Vol. 123, No. 26, pp. 4089-4100 on June 26th, 2014.

Chapter 2

T cell-specific expression of a Bcl-2 BH3 domain mutant transgene inhibits negative selection and causes autoimmunity

Summary

T cell tolerance is thought to involve dominant tolerance, mediated by T_{reg} cells, and negative selection involving apoptosis of autoreactive thymocytes. While the importance of dominant tolerance is firmly established, evidence supporting an essential role for negative selection is limited. $Aire^{-/-}$ mice, defective in negative selection against tissue-restricted antigens, develop multi-organ autoimmunity. However, loss of Bim, a pro-apoptotic Bcl-2 family protein essential for thymocyte apoptosis, rarely results in autoimmunity on the C57BL/6 background. $Bim^{-/-}/Puma^{-/-}$ mice do develop autoimmunity, but non-T cells may play a role, as mice with T cell-specific over-expression of Bcl-2, that blocks Bim and Puma, are largely normal. The nuclear receptor Nur77 has also been implicated in negative selection, but its mode of action remains unclear. Nur77 can associate with Bcl-2 to expose its BH3 domain, potentially turning Bcl-2 into a pro-apoptotic molecule. Here, we report that T cell-specific expression of a Bcl-2 BH3 mutant transgene results in enhanced rescue of thymocytes from negative selection. Concomitantly, T_{reg} development is increased. However, aged BH3 mutant mice progressively accumulate activated, autoreactive T cells, culminating in the development of multi-organ autoimmunity and lethality. These data provide strong evidence that apoptosis accompanying negative selection is crucial for establishing T cell tolerance.

Introduction

Maintenance of T cell self-tolerance is essential for the prevention of autoimmune diseases. A series of mechanisms are thought to be involved in establishing T cell tolerance, the first of which is deletion of potentially self-reactive T cells in the thymus. During T cell development, autoreactive T cells bind self-peptide presented on major histocompatibility complex (MHC) molecules with high affinity and receive strong T cell receptor (TCR) signals that trigger apoptosis and clonal deletion. Some of these cells can also differentiate down an alternative pathway to a harmless lineage, such as T regulatory cells (T_{reg}), or also CD8 $\alpha\alpha$ and invariant NK T cells³³. The importance of this process, termed negative selection, in establishing self-tolerance and preventing autoimmunity was best demonstrated in studies of mice deficient in the autoimmune regulator (AIRE) gene. These mice develop a multi-organ autoimmune disease as the result of defective deletion of auto-reactive T cells specific to tissue-restricted antigens (TRA), which would normally be expressed by medullary thymic epithelial cells under the control of AIRE³⁶. Humans with loss of AIRE function develop similar disease pathology^{52,53}. However, additional models that implicate a breakdown in negative selection as the driving force behind the development of autoimmune disease are lacking³⁴. This might suggest that under most circumstances, subsequent tolerizing mechanisms in the peripheral compartments such as anergy or T_{reg} -mediated suppression are sufficient to compensate for defective thymic deletion. Even in the case of AIRE-deficiency, impaired tolerization of CD4⁺ T cells in the periphery by extra-thymic AIRE expressing cells likely contributes to the development of autoimmunity³⁷. This has cast doubt on the relative importance of negative selection for maintaining immunological self-tolerance.

Apoptosis of thymocytes during negative selection is dependent on the mitochondrial apoptosis pathway regulated by the Bcl-2 family of proteins⁵⁴. In particular, the pro-apoptotic, Bcl-2 homology domain 3 (BH3) only protein, Bim, which is upregulated in response to strong TCR signals, is essential for this process. Bim and other BH3-only proteins are thought to promote apoptosis by binding and inhibiting anti-apoptotic Bcl-2 proteins (such as Bcl-2, Bcl-X_L and Mcl-1) that directly suppress downstream effectors Bax and Bak, which are responsible for disrupting mitochondrial membrane integrity⁶. Bim-deficiency has been shown to cause defective thymocyte apoptosis in several TCR transgenic (HY, OTI, OTII) models of negative selection^{15,38,55,56}. However, this apoptotic defect does not lead to emergence of autoreactive mature T cells except in the case of negative selection to TRA⁵⁶⁻⁵⁸. Interestingly, Bim^{-/-} mice on a mixed 129/Sv X C57BL/6 background develop a systemic lupus erythematosus (SLE)-like autoimmune disease, indicative of a breakdown in tolerance³⁹. However, loss of Bim in other immune compartments likely contributes to the disease, especially B cells, which are important mediators of SLE pathology. This phenotype was greatly ameliorated on the autoimmune-resistant C57BL/6 background and is distinct from the multi-organ, T cell dominant disease found in AIRE-deficient mice^{40,59}. Recently, a study showed that additional loss of Puma, another BH3-only protein, enhanced the Bim^{-/-} thymic deletion defect and led to the development of immune pathology more similar to that found in AIRE-deficient mice⁴⁴. T cells from these mice were able to transfer the disease, lending support to it being T cell-driven. However, the role of non-T cells in these mice cannot be excluded due to germline deletion of Puma and Bim. In addition, T-cell specific over-expression of Bcl-2, which can inhibit both Bim and Puma⁶⁰, does not lead to autoimmunity^{42,43}, suggesting that a defect in T cells alone may be insufficient to cause disease. Alternatively, redundant negative selection pathways involving Bim, Puma and the Nur77 family members (see below) may not allow Bcl-2 over-expression to block all pathways leading to negative selection.

In addition to Bim, the Nur77 family of orphan steroid receptors, which includes Nur77, Nor-1 and Nurr1, has also been implicated in apoptosis accompanying negative selection. Nur77 expression, like that of Bim, is induced by strong TCR signals that result in negative selection. Low expression of both proteins has been correlated with defective clonal deletion in Non-obese Diabetic (NOD) mice^{61,62}. Furthermore, T cell-specific over-expression of Nur77, or Nor-1, results in massive apoptosis of thymocytes¹⁷. Expression of a dominant negative Nur77 protein that can block all family members results in inhibition of apoptosis in the F5 and HY TCR transgenic models of negative selection^{18,63}. Deficiency in Nur77 alone results in moderate inhibition of apoptosis accompanying negative selection in the OTII and BDC2.5 TCR transgenic models¹⁹. Interestingly, the Nur77 family has also been shown to transcriptionally regulate Foxp3 expression and deletion of all three Nur77 family members results in impaired T_{reg} cell production and development of a quick onset, severe autoimmune disease resembling that of Foxp3-deficient mice²¹. Therefore, Nur77 could contribute to the maintenance of immunological tolerance both by facilitating deletion of autoreactive thymocytes and ensuring production of T_{reg} cells. The mechanism by which Nur77 and its family members promote thymocyte apoptosis has long been under investigation. We and others showed that strong TCR signals induce the translocation of Nur77 and Nor-1

from the nucleus to the mitochondria in thymocytes, a phenomenon first reported to occur in cancer cells in response to apoptotic stimuli^{26,27,29}. At the mitochondria, Nur77 and Nor-1 bind Bcl-2 and cause exposure of Bcl-2's BH3 domain, the "death" domain of the Bcl-2 family of proteins. Bcl-2 BH3 exposure correlates with thymocyte apoptosis in TCR-stimulated thymocytes and HY and F5 TCR transgenic thymocytes undergoing negative selection²⁶. BH3-exposed Bcl-2 might act similarly to BH3-only protein Bim to bind and inhibit other anti-apoptotic Bcl-2 proteins, such as Bcl-X_L, as was demonstrated in cancer cells³⁰. It is thus possible that Nur77 converts Bcl-2 to a pro-apoptotic effector during negative selection, preventing over-expression of Bcl-2 from completely blocking negative selection.

Here, we generated a T cell-specific Bcl-2 BH3 mutant transgenic mouse, in which its purported proapoptotic BH3 function should be abolished by changing its conserved BH3 residues, GDD, to alanines⁶⁴. We found that over-expression of both wild-type and BH3 mutant Bcl-2 efficiently rescued thymocyte apoptosis in two TCR transgenic models of negative selection. However, the BH3 mutant transgene more effectively blocked TCR-induced thymocyte apoptosis *in vitro* and better rescued high affinity TCR clones from deletion in polyclonal systems *in vivo*. Interestingly, BH3 mutant transgenic mice, in contrast to the reported wild-type Bcl-2 transgenic mouse phenotype, developed T cell-driven multi-organ autoimmune pathology. Thus, we have found evidence that a breakdown in thymocyte apoptosis during negative selection is indeed sufficient to cause autoimmune disease.

Results

Generation of T cell-specific Bcl-2 BH3 domain mutant transgenic mice

To test the *in vivo* relevance of the Bcl-2 BH3 domain pro-apoptotic function in thymocyte apoptosis, we created T cell-specific Bcl-2 BH3 mutant transgenic mice. Three amino acid residues critical for BH3 domain pro-apoptotic function were mutated to alanine (Figure 1A) and the mutated human Bcl-2 transgene was expressed under the control of the *cd4* regulatory elements^{65,66}. The "BH3" transgenic mice (BH3 Tg) were generated on the C57BL/6 background and two founder lines were chosen for analysis, BH3 A and BH3 B. For evaluation, the BH3 Tg mice were compared to the T cell-specific wild-type human Bcl-2 strain, *lck^{Pr}-bcl-2*, also on the C57BL/6 background, referred to here as "Bcl-2 Tg"⁴³.

By intracellular staining with an antibody that detects both mouse and human Bcl-2, we observed that Bcl-2 expression was significantly increased in BH3 Tg thymocytes compared to wild-type controls (Figure 1B). Although the transgene is expressed by the *cd4* promoter, increased Bcl-2 expression was detected in DN (CD4⁻ CD8⁻ double negative) thymocytes as early as CD25⁺ CD44⁻ DN3 (Figure 1B). This was very similar to the expression pattern of the *lck*-driven wild-type Bcl-2 transgene. Additionally, the level of Bcl-2 upregulation in BH3 Tg versus Bcl-2 Tg thymocytes was very similar in DN, DP and CD4 single positive (CD4 SP) thymocytes. Only slightly higher expression of the BH3 transgene was noted in DP and CD4 SP cells. As expected, BH3 transgene expression was downregulated in CD8 single positive (CD8 SP) thymocytes due to loss of CD4 expression (Figure 1B). Lymph node CD8 T cells further downregulated BH3 transgene expression, while both BH3 Tg and Bcl-2 Tg CD4

T cells maintained similarly high levels (Figure 1B). Interestingly, we observed a compensatory downregulation of endogenous mouse Bcl-2 in the thymus, lymph nodes and spleen of both BH3 and Bcl-2 Tg mice (Figure 1C). Thus, we do not have to consider a contribution of endogenous mouse Bcl-2 to the phenotype of these mice. Due to close similarity in transgene expression level and pattern in the two BH3 Tg lines, BH3 A and BH3 B, we used these mice interchangeably throughout our study (data not shown). In both BH3 Tg lines, we observed a decreased proportion of DP thymocytes and an increased proportion of SP thymocytes compared to wild-type littermate controls (Figure 1D). However, only the absolute SP cell numbers were increased, especially for CD4 SP cells, while the DP cell number was comparable to wild-type controls (Figure 1E). This is largely similar to the thymocyte distribution found in the wild-type Bcl-2 Tg mice⁴³. As expected, the peripheral mature T cell compartment reflects the increased SP thymocyte numbers in both the BH3 Tg and Bcl-2 Tg mice (Figure 1F). Relative to wild-type mice, the CD4 to CD8 T cell ratio is therefore somewhat skewed in favor of CD4 T cells in the BH3 Tg mice and CD8 T cells in the Bcl-2 Tg mice. Hence, differential expression in the CD8 compartment is one caveat to comparison of BH3 and Bcl-2 transgenic mice. However, similar transgene expression levels in the CD4 compartment should allow fair comparison of DP, CD4 SP and CD4 T cell phenotypes.

Defective negative selection in BH3 Tg mice

A defect in thymocyte apoptosis accompanying negative selection could potentially contribute to increased SP thymocyte and mature T cell numbers in the BH3 and Bcl-2 Tg mice. However, while Bcl-2 transgene expression was previously shown to safeguard thymocytes against a variety of apoptotic stimuli, thymocytes were inefficiently protected from apoptosis during negative selection^{38,43,67}. To test whether BH3 Tg thymocytes might be better protected, we first assessed thymocyte apoptosis in response to strong TCR stimuli that mimic negative selection *in vitro*. In response to anti-CD3 and anti-CD28 antibody stimulation, BH3 transgene expression substantially decreased apoptosis of thymocytes compared to wild-type controls (Figure 2A). In addition, BH3 Tg thymocytes showed significantly increased resistance to apoptosis compared to Bcl-2 Tg thymocytes.

To test whether the BH3 transgene is also better able to block apoptosis during negative selection *in vivo*, we examined apoptosis of F5 TCR transgenic thymocytes in BH3 Tg mice crossed to F5 mice (BH3/F5)^{68,69}. We also examined Bcl-2/F5 mice for comparison. In response to injection with the F5 TCR-specific peptide, NP, F5 DP thymocytes undergo massive apoptosis⁶⁹. Strikingly, however, expression of either the BH3 or Bcl-2 transgene almost completely inhibited thymocyte death, with thymocyte numbers only marginally decreased compared to PBS-injected control mice after 48 hours of treatment (Figure 2B). Analysis of thymocyte proportions indicated a significant rescue of the DP thymocyte population (Figure 2C). We sought to increase the sensitivity of the assay by using a previously described *ex vivo* thymic slice approach⁷⁰. This assay allows assessment of apoptosis over a more acute timeline, free of possible cytotoxic effects inherent in the NP injection model⁶⁹. Here, again, we saw near complete and equivalent protection of BH3 and Bcl-2 Tg thymocytes from apoptosis in response to NP peptide treatment over a 36-hour time course (Figure 2D). Thymocytes

of all genotypes were similarly activated as measured by upregulation of CD69 (Figure 2D). We also performed an analogous thymic slice assay comparing apoptosis of thymocytes from female BH3 or Bcl-2 Tg mice crossed to HY TCR transgenic mice (BH3/HY or Bcl-2/HY, respectively) in response to the HY TCR-specific peptide, Smcy. Remarkably, just as in the F5 model, thymocytes expressing either transgene were significantly protected from apoptosis (Figure 2E). Hence, we conclude that Bcl-2 over-expression very efficiently inhibits thymocyte apoptosis accompanying negative selection in these models and this potent blockade could not be enhanced by expression of a BH3 mutant form of Bcl-2.

BH3 transgene enhances rescue of high affinity TCR clones in a polyclonal system

We next sought to test whether over-expression of Bcl-2 is sufficient to block negative selection in a normal, polyclonal system and if in this context, the BH3 transgene might exhibit enhanced anti-apoptotic activity. To investigate this, we employed the Nur77^{GFP} BAC transgenic reporter mouse system, in which GFP is inserted into the start site of the Nur77 transgene¹⁵. Studies of these mice have clearly demonstrated that the level of Nur77 expression in T cells directly correlates with the intensity of the TCR signals received¹⁵. During thymocyte selection, cells receiving strong TCR signals are destined to die by negative selection while cells receiving very weak to no signal die by neglect. Hence, the post-selection thymocyte pool is largely comprised of cells expressing an intermediate level of GFP, reflecting their receipt of moderate, positively selecting TCR signals. In Bim^{-/-} X Nur77^{GFP} mice, however, a significant number of post-selection thymocytes express high levels of GFP, indicating that high affinity TCR clones are rescued from negative selection in the absence of Bim⁷¹. This rescue was also reflected by an increase in the total number of post-selection DP and SP thymocytes. When we assessed GFP expression of post-selection thymocytes in BH3/Nur77^{GFP} and Bcl-2/Nur77^{GFP} mice, we observed a strikingly similar phenotype. A large population of GFP-high post-selection DP thymocytes was rescued with the expression of either transgene (Figure 3A). As in Bim^{-/-}/Nur77^{GFP} mice, the proportion of post-selection DP thymocytes was consequently increased compared to wild-type mice, reflecting the rescue of these cells from deletion (Figure 3A).

To quantify this effect, we defined GFP-high (GFPhi) cells as those expressing GFP at a level greater than the mean fluorescence intensity (M.F.I.) of wild-type post-selection DP cells; this definition allowed for the most consistent gating across independent experiments (Figure 3A). The percent increase in GFPhi cells over wild-type is indicative of the proportion of cells rescued from negative selection by transgene expression. Interestingly, a significantly greater increase in the percentage of GFPhi post-selection DP cells was observed in BH3/Nur77^{GFP} versus Bcl-2/Nur77^{GFP} mice, suggesting that the BH3 transgene might indeed have an enhanced ability over wild-type Bcl-2 to rescue high affinity TCR clones from apoptosis (Figure 3B). No increase was observed in GFP-intermediate (GFPint) cells, indicating that the GFPhi population encompasses all rescued cells and that BH3 and Bcl-2 transgene expression, similar to Bim-deficiency, only rescues high affinity clones and therefore does not seem to enhance positive selection (Figures 3A-B)⁷¹. BH3 and Bcl-2 transgene expression also rescued GFPhi CD4 and CD8 SP thymocytes from apoptosis. This likely indicates

rescue of high affinity clones from deletion in response to self-antigens presented in the medulla and possibly rescue of DP cells that have differentiated into SP thymocytes. A greater percentage of GFPhi CD4 SP cells were rescued in BH3 Tg versus Bcl-2 Tg mice (Figure 3C). Consistent with the BH3 transgene being downregulated in CD8 SP cells, BH3 expression rescued a smaller percentage of GFPhi CD8 SP thymocytes compared to CD4 SP (Figure 3D versus 3C). Collectively, these data demonstrate that Bcl-2 over-expression causes a significant defect in thymocyte negative selection even in a normal, polyclonal system and in this context, the BH3 mutant protein rescues significantly more cells from death than wild-type Bcl-2.

Defective superantigen negative selection in BH3 Tg mice

Endogenous superantigen negative selection is another polyclonal model of clonal deletion whereby specific Vb TCR-expressing T cells are deleted through interaction of their specific Vb chain with endogenously-expressed superantigen bound to class II MHC (usually I-E) on antigen presenting cells⁷². Whether Bcl-2 over-expression can inhibit negative selection against endogenous superantigens is controversial. In one study, Bcl-2 transgene expression did not block superantigen negative selection⁴³ while another group clearly saw a rescue⁷³. Yet another study reported that cells were rescued from superantigen-mediated deletion in the Bcl-2 transgenic thymus, but not in the transgenic lymph nodes⁶⁷. To assess endogenous superantigen negative selection in BH3 Tg mice, we crossed them to the class II MHC I-E expressing CBA/J strain of mice. CBA/J mice express superantigens that delete T cells expressing Vb3, 5, 6, 7, 8.1, 9 and 11, but not Vb8.2⁷². We examined the proportions of Vb5, Vb6, Vb8.1/8.2 and Vb11 expressing DP and SP thymocytes in BH3 Tg X CBA/J mice and their wild-type littermate controls as well as in Bcl-2 Tg X CBA/J mice. As shown in Figure 4, the proportions of Vb5, Vb6 and Vb11, but not Vb8.1/8.2, expressing cells decreased between the DP and SP stage in non-transgenic mice, indicative of superantigen negative selection. In both BH3 and Bcl-2 Tg mice, however, increased proportions of Vb5, Vb6 and Vb11 expressing SP thymocytes were observed, suggesting that expression of either transgene can block superantigen-mediated deletion. The BH3 transgene rescued CD8 SP thymocytes less efficiently than the wild-type Bcl-2 transgene, reflective of the relative levels of transgenic protein in the CD8 compartment. CD4 SP thymocytes were rescued to a similar extent by both transgenes, however modestly better by the BH3 transgene for Vb5⁺ and Vb6⁺ cells. Interestingly, we observed a significant increase in Vb5⁺ and Vb6⁺ (but not Vb11⁺) DP thymocytes in the BH3 Tg mice that was not seen in the Bcl-2 Tg mice. The pattern of rescue in this model parallels that observed in the Nur77^{GFP} polyclonal system. Together, these data are consistent with the notion that the BH3 Tg mice exhibit an enhanced ability to rescue thymocytes from negative selection.

Alternative tolerizing mechanisms are increased in BH3 Tg mice

Our results demonstrate that T cell-specific over-expression of wild-type Bcl-2 clearly results in a rescue of cells receiving strong TCR signals, which would normally undergo apoptosis, yet aged Bcl-2 Tg mice were not reported to develop autoimmune pathology⁴². Hence, we sought to investigate the fate of the rescued, high affinity T cell clones in the Bcl-2 Tg and BH3 Tg mice. We observed that the GFP mean fluorescence

intensity was higher in lymph node T cells from both BH3/Nur77^{GFP} and Bcl-2/Nur77^{GFP} mice (Figure 5A). Interestingly, GFP levels were higher in Bcl-2 Tg versus BH3 Tg T cells, even for CD4⁺ T cells. The reason for this is unclear, but it may be due to distinct T cell repertoires in these two lines of mice that lead to differences in T cell signaling. T_{reg} cells have self-reactive TCRs and express a high level of GFP, however T_{reg} cells did not account for the majority of the GFP-high CD4 T cells (data not shown). This indicates that conventional T cells with higher than normal TCR affinity are permitted to migrate into the periphery. Since presumably autoreactive T cells are found in the periphery, mechanisms must exist to keep these cells in check. One mechanism might be the production of more T_{reg} cells to mediate suppression of the autoreactive cells³³. Interestingly, we observed that GFP-high CD4 SP thymocytes rescued from negative selection express GFP at a very similar level to T_{reg} cells (Figure 5B). Since high TCR affinity contributes to T_{reg} development³³, we hypothesized that more thymocytes might be directed down this pathway in BH3 and Bcl-2 Tg mice. Indeed, we found greater Foxp3⁺ T_{reg} cell proportions and numbers in the both the thymus and lymph nodes of BH3 and Bcl-2 Tg mice (Figure 5C). BH3 Tg mice had a more significant increase in peripheral T_{reg} numbers compared to Bcl-2 Tg mice (3-fold versus 2-fold), mirroring the higher mature CD4 T cell numbers in these mice. Most of the increase in total T_{reg} number was caused by an increase in CD25⁻, and not CD25⁺ cells. CD25⁻ T_{reg} cells are thought to be derived from diverted, potentially autoreactive thymocytes and are functional, but have reduced suppressive capacity compared to CD25⁺ T_{reg} cells⁵⁵. Despite the increase in total T_{reg} number in BH3 and Bcl-2 Tg mice, the ratio of CD25⁺ T_{reg} cells to total CD4 T cells was similar to wild-type mice (Figure 5C). Thus, some of the rescued high affinity clones appear to be diverted down the CD25⁻ T_{reg} pathway, but not the CD25⁺ T_{reg} pathway. Intriguingly, consistent with this, we observed that CD25⁻ T_{reg} cells express higher levels of GFP than CD25⁺ T_{reg} cells, at a similar level to the highest GFP-expressing BH3 Tg clones (Figure 5D). Another mechanism employed to maintain peripheral tolerance is the induction of T cell anergy upon antigen encounter. In addition to increased T_{reg} cells, we also observed a higher proportion of previously activated, anergic CD4 T cells in BH3 and Bcl-2 Tg mice marked by upregulation of the markers FR4 and CD73 in CD44^{hi} T cells (Figure 5E-F)⁷⁴. Therefore, increased diversion to the T_{reg} lineage/T_{reg} suppression and CD4 T cell anergy might cooperate to suppress the emergence of autoimmunity in these mice.

BH3 Tg mice have a shortened lifespan and lymphoid hyperplasia

Despite similarly engaging alternative mechanisms to promote tolerance, BH3 Tg mice, unlike Bcl-2 Tg mice, have a considerably shortened life-span, with a median length of survival of about 50 weeks for both BH3 A and BH3 B lines (Figure 6A). Most mice were sacrificed due to general deterioration of health, especially dramatic weight-loss, but some mice of the BH3 A line were sacrificed due to a severe inflammatory skin condition. No Bcl-2 Tg mice had to be sacrificed over a 60-week period. Upon examination of a large cohort of mice between 45 and 60 weeks-of-age, we found that all BH3 Tg mice displayed severe splenomegaly and lymphadenopathy and had significantly increased numbers of both T cells and B cells, especially in the mesenteric lymph nodes (MLN) and spleen (Figure 6B-6C). In comparison, Bcl-2 Tg lymphoid organs were only mildly enlarged, but a significant increase in T cell numbers was

observed. We also observed a dramatic increase in CD3⁺ B220⁺ Myeloid/other cells in the MLN and spleen of BH3 Tg, but not Bcl-2 Tg mice (Figure 6D). A large proportion of these cells were Ly6C^{lo} CD11b⁺ neutrophils, indicative of increased inflammation in these mice (Figure 6E).

BH3 Tg mice accumulate activated, autoreactive T cells

CD4 and CD8 T cells in aged BH3 Tg mice were highly activated, as indicated by increased CD44 expression and decreased CD62L expression, reminiscent of the phenotype of the disease-promoting T cells in AIRE-deficient mice (Figure 7A;³⁶). We also observed a significant increase in CD44^{hi}/CD62L^{hi} central memory CD8 T cells. An increase in activated T cells was also sometimes observed in Bcl-2 Tg mice, but the phenotype was greatly attenuated. While we did not observe an increase in activated T cells in young mice (data not shown), starting as early as 20 weeks-of-age, there was significant accumulation of activated T cells in the peripheral blood of BH3 Tg mice (Figure 7B). This increase became very dramatic and nearly ubiquitous in 40 to 50-week-old mice. In comparison, only a couple Bcl-2 Tg mice at 40 to 50 weeks were found to have increased activated T cells. To directly test the possible autoreactivity of BH3 Tg T cells, we stimulated T cells with syngeneic or allogeneic irradiated splenocytes and measured T cell proliferation in a mixed lymphocyte reaction (MLR). All T cells were able to proliferate in response to allogeneic stimulators, although CD4 T cells proliferated less than CD8 T cells and the presence of Bcl-2 further reduced proliferation (Figure 7C, left panel). This datum is consistent with the known inhibitory effect of Bcl-2 on cell cycle progression^{75,76}. When presented with syngeneic stimulators, little proliferation was observed for all the Tg and wild-type CD4 T cells, most likely due to a combination of the lack of ability of CD4 T cells to proliferate in this system and the inhibitory effect of Bcl-2 on cell cycle progression. In contrast, CD8⁺ BH3 Tg T cells exhibited significant proliferation in response to syngeneic stimulators, suggesting that the BH3 Tg mice contain autoreactive T cells (Figure 7C). Despite our inability to detect autoreactive CD4 T cells in BH3 Tg mice by MLR, we found that the activated CD4 T cells in aged mice appear to have increased effector function, as splenic CD4 T cells from 50-week-old mice secrete increased amounts of IFN γ (Figure 8A). Only IFN γ and not IL-17 or IL-4 was increased, suggesting that the activated T cells are Th1 polarized (Figure 8A). A few BH3 Tg mice also exhibited a significant increase in anti-nuclear antibodies (ANA), demonstrating increased B cell activation against self-tissues, likely due to increased T cell help (Figure 8B). However, for the majority of the BH3 Tg mice, the levels were not appreciably higher than wild-type or Bcl-2 Tg mice, perhaps consistent with the autoimmune pathology in these mice being more predominantly T cell- rather than B cell-driven. Also consistent with this, we noted significant infiltration of lymphocytes into the liver, lung and kidney of BH3 but not Bcl-2 Tg mice (Figure 8C). In a few mice with high levels of ANA, significant disruption of kidney morphology was observed, reminiscent of glomerulonephritis (Figure 8C). Aged BH3 Tg mice also had significantly increased numbers of both CD25⁺ and CD25⁺ T_{reg} cells in their lymphoid organs, making up approximately 50% of all CD4 T cells (Figures 8D-E). This dramatic increase in T_{reg} cells was still apparently insufficient to inhibit autoimmunity in these mice.

Discussion

Bcl-2 family proteins and the Nur77 family of orphan steroid receptors are critical mediators of thymocyte apoptosis during negative selection. However, whether thymocyte deletion is necessary to maintain immunological self-tolerance remains under debate. Here, we find that T cell-specific over-expression of a Bcl-2 BH3 mutant transgene results in T cell-driven multi-organ autoimmunity, demonstrating that a defect in thymic deletion is indeed sufficient to cause a breakdown in tolerance.

Consistent with previous observations, we found that T cell-specific over-expression of wild-type Bcl-2 did not cause significant autoimmune pathology⁴². However, our results show that Bcl-2 Tg mice may develop autoimmunity at low penetrance, as some mice exhibited a modest accumulation of activated T cells. In contrast, expression of a Bcl-2 BH3 mutant transgene significantly increased the severity of the disease and resulted in greater than 70% lethality by one year-of-age. A significant accumulation of activated T cells was observed starting at 20 to 30 weeks-of-age and ultimately resulted in massive lymphoid hyperplasia, inflammation, lymphocyte infiltration into non-lymphoid organs and some auto-antibody accumulation. Our data in the Nur77^{GFP} and superantigen mouse models indicate that the Bcl-2 BH3 mutant is better able to rescue autoreactive thymocytes from negative selection than wild-type Bcl-2. Abolishing the Nur77-dependent pro-apoptotic function of Bcl-2 by BH3 mutation might therefore enhance the anti-apoptotic effects of Bcl-2 over-expression. Alternatively, it is also possible that slightly higher BH3 transgene expression levels in DP and CD4 SP cells could be responsible for this phenotype, although the levels of transgene expression do not necessarily correlate with GFP expression as demonstrated by the higher GFP M.F.I. in CD4⁺ T cells of Bcl-2 over BH3 Tg mice (Figure 5A). Cause set aside, a greater defect in negative selection could allow a threshold to be crossed in which other mechanisms of maintaining tolerance are no longer sufficient to prevent disease. This would be consistent with the recent study comparing the severity of disease in Bim^{-/-} versus Bim^{-/-} Puma^{-/-} mice. Bim^{-/-} mice on the C57BL/6 background were reported to have attenuated autoimmunity and 80% of them still survive at 70 weeks-of-age^{40,41}. Additional loss of Puma, however, enhanced the thymic deletion defect of Bim^{-/-} mice and resulted in more severe, T cell-driven multi-organ autoimmune pathology⁴⁴. It remains to be seen if loss of Bim and Puma in T cells alone would result in the same phenotype. Our observations in a T cell-specific model provide strong evidence that a severe thymic clonal deletion defect is sufficient to cause autoimmune pathology.

Despite the large disparity in autoimmune phenotype, we found that both BH3 and Bcl-2 Tg thymocytes were significantly protected from apoptosis in TCR transgenic models of negative selection. Previously, Bcl-2 over-expression was directly compared with Bim-deficiency in the HY model and Bcl-2 over-expression fell significantly short of Bim-deficiency in rescuing thymocytes from deletion³⁸. However, in our thymic slice assay of HY TCR-mediated deletion, we find that Bcl-2 over-expression almost completely blocks thymocyte death. Similarly, over-expression of Bcl-2 in F5 TCR transgenic mice dramatically blocked thymocyte deletion by thymic slice assay and *in vivo* peptide injection. One possible explanation for the discrepancy between our results and previously published observations might be the negative selection models used. Apoptosis of HY TCR transgenic mice is notoriously difficult to block due to accelerated

T cell development and early expression of the transgenic TCR⁷⁷. Indeed, using thymic organ culture, another group has previously reported a significant inhibition of negative selection by Bcl-2 in the F5 model⁷⁸. Regardless, however, we have demonstrated that Bcl-2 over-expression has a potent anti-apoptotic effect on negative selection in a normal, polyclonal system. Mirroring observations in Bim^{-/-}/Nur77^{GFP} mice⁷¹, we observed a striking increase in GFP-high post-selection thymocytes in Bcl-2/Nur77^{GFP} mice, indicating a significant rescue of high affinity TCR clones from deletion. Here, over-expression of the BH3 mutant transgene resulted in substantially greater rescue of GFP-high DP and CD4 SP thymocytes compared to over-expression of wild-type Bcl-2. BH3 transgene expression also enhanced rescue of $\nu\beta 5$ and $\nu\beta 6$ DP and CD4 SP thymocytes from deletion in a polyclonal superantigen model of negative selection. Increased rescue of these cells in the thymus might lead to the development of a more self-reactive T cell repertoire, accelerating the expansion of activated T cells in BH3 Tg mice.

Differential expression of the BH3 versus wild-type Bcl-2 transgenes in the CD8 T cell compartment might also contribute to the development of autoimmunity in BH3 Tg, but not Bcl-2 Tg mice. CD4-driven transgene expression in the BH3 transgenic mice preferentially enhances CD4 T cell seeding of the peripheral lymphoid organs. BH3 Tg mice have about a 3:1 ratio of CD4 to CD8 T cells in the lymph nodes compared to a 2:1 ratio in wild-type mice. In Bcl-2 Tg mice, the proportions are skewed in the opposite direction, resulting in a 1:1 ratio. This is likely due to a combination of transgene-biased rescue of cells from negative selection and increased survival of transgene-expressing T cells. BH3 Tg mice are therefore expected to have increased numbers of autoreactive CD4 T cells, which as key mediators of autoimmunity, could be responsible for the acceleration of disease in these mice⁷⁹. Alternatively, as Bcl-2 over-expression reduces the ability of T cells to proliferate^{75,76}, downregulation of the BH3 transgene in CD8 T cells might enhance their functional capacity over that of Bcl-2 Tg CD8 T cells. As CD8 T cells play a well-documented role in T cell-mediated, tissue-specific autoimmune diseases (i.e. multiple sclerosis and diabetes), more functionally competent autoreactive CD8 T cells in the BH3 Tg mice might contribute to the development of disease⁸⁰.

In response to defective clonal deletion in the thymus, we observed compensatory induction of alternative tolerizing mechanisms that might prevent earlier onset of autoimmunity in BH3 Tg mice. An increase in the proportion and number of T_{reg} cells was noted in both the thymus and lymph nodes of young mice. Interestingly, CD25⁻ T_{reg} cells accounted for most of the increase in total T_{reg} numbers and these cells have been reported to have reduced suppressive function⁵⁵. These CD25⁻ T_{reg} cells are thought to be derived from diverted, autoreactive cells that were not deleted in the thymus. Consistent with this, CD25⁻ T_{reg} cells in the thymus expressed GFP at a higher level than CD25⁺ T_{reg} cells and at a similar level to the most GFP-high naïve CD4 SP cells in the BH3/Nur77^{GFP} mice. Hence, diverting a portion of these GFP-high autoreactive cells to this lineage might be the more effective tolerizing strategy. Notably, our observation that CD25⁺ T_{reg} proportions were not increased in the BH3 Tg mice suggests that T cells with self-reactive TCRs are not automatically committed to the T_{reg} lineage and that there is likely a saturated niche for CD25⁺ T_{reg} development determined by availability of other factors, such as IL-2 and IL-15³³. In addition to increased T_{reg} cells, we observed a 2-fold increase in the proportion of anergic CD4 T cells in the BH3

and Bcl-2 Tg mice. This was similarly observed in Bim^{-/-} mice⁷¹. Induction of anergy could cooperate with diversion to the T_{reg} lineage/T_{reg} suppression to inhibit T cell-mediated promotion of autoimmunity and prevent early onset of disease. However, differential induction of these tolerizing mechanisms was not observed in BH3 Tg versus Bcl-2 Tg mice and therefore cannot account for the eventual development of autoimmunity in BH3 Tg, but not Bcl-2 Tg mice. Interestingly, in aged BH3 Tg mice, T_{reg} cells made up almost 50% of the CD4 T cell pool in mice displaying autoimmune pathology. However, this protective response was still apparently insufficient to suppress autoimmunity.

Finally, the enhanced ability of the Bcl-2 BH3 mutant transgene to rescue autoreactive thymocytes from apoptosis compared to wild-type Bcl-2 could suggest that the Bcl-2 BH3 domain might indeed have some pro-apoptotic function. This finding may provide *in vivo* support for the notion that Nur77 promotes thymocyte apoptosis through conversion of Bcl-2 to a pro-apoptotic effector. However, this still needs to be further confirmed in a knock-in model. Additionally, analysis of a Bcl-2 protein that cannot interact with Nur77 would also be required to confirm that Bcl-2 pro-apoptotic function is Nur77-dependent. Intriguingly, it has previously been suggested that Nur77 and Bim might function in the same pathway, as Bim-deficiency was unable to synergize with Nur77-deficiency to inhibit thymocyte apoptosis in the OTII TCR transgenic model of negative selection¹⁹. If Bcl-2 conversion is indeed critical for Nur77-mediated thymocyte apoptosis, this would support the idea that Nur77 and Bcl-2 family members come together at the mitochondria to cooperatively regulate cell death during negative selection.

Materials and Methods

Mice

The mutant allele for the Bcl-2 BH3 mutant transgenic mice (BH3 Tg) was generated by QuikChange site-directed mutagenesis (Agilent) from a pCI-human Bcl-2 vector using the following primers: BH3 forward CTGACCCTCCGCCAGGCCGCGGCCGCTTCTCCCGCCGCTACCGC, BH3 reverse GCGGTAGCGGCGGGAGAAGGCGGCCGCGGCCTGGCGGAGGGTCAG. The nucleotide sequence of amino acids 101-103 was changed from GGCGACGAC to GCGGCCGCC, creating a restriction enzyme site change from BglI to NotI. The mutant transgene was then cloned into the XhoI site of the pTG4 construct^{65,66}. The pTG4 construct was then modified to include the *cd4* locus control region (LCR). PCR amplification of the 1kb LCR sequence from C57BL/6 genomic DNA was performed with the following primers: LCR forward GACATCGATAGCTAGCACACGCCGGTAAGCCCATTCCCCACGC, LCR reverse GACATCGATGCGGTACCGATCCCAACCAAACTGCGGCCCTTTCA. The LCR was added to the 5' end of the CD4 silencer at a ClaI restriction site. The transgenic mice were generated on the C57BL/6 background using standard procedures. Founders were identified by PCR genotyping with the following primers: HGH forward GACACAACTCACACAACGATGACGC, HGH reverse ATGCCTGGAATCCCAACAACCTCGG. The presence of the BH3 GDD to AAA mutation in the incorporated transgene was confirmed by PCR amplification of the transgene

from genomic DNA and successful digestion with NotI, but not BglI enzyme. The BH3 Tg mice were compared with the T cell-specific wild-type Bcl-2 transgenic line *lck^{Pr}-bcl-2*⁴³, which have been back-crossed to C57BL/6 for more than 10 generations, referred to here as Bcl-2 Tg. The BH3 and Bcl-2 Tg mice were crossed with the following transgenic lines: Nur77^{GFP}¹⁵, F5 TCR⁶⁸, and HY TCR⁸¹. All mice were on the C57BL/6 background. All experimental protocols involving animals were approved by the UC Berkeley Animal Care and Use Committee.

Flow Cytometry

Lymphoid organs were dissociated through 40µm cell strainers to obtain a single cell suspension and a red blood cell lysis was performed. Cells were surface stained with fluorochrome-conjugated antibodies (eBioscience, BD Biosciences and Tonbo Biosciences) in 1% fetal bovine serum in PBS. For Annexin V staining, after surface staining, cells were stained with FITC-conjugated Annexin V (BD Bioscience) in 1X Annexin V binding buffer (10X: 0.1M HEPES, pH 7.4; 1.4M NaCl; 25mM CaCl₂). For intracellular staining, prior to surface staining, cells were stained with Tonbo Ghost Dye (Tonbo Biosciences) to label dead cells, then fixed and permeabilized with a Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). For Bcl-2 intracellular staining, cells were then stained with FITC-conjugated anti-human Bcl-2 (Clone 124, Dako) or a FITC-conjugated mouse IgG1 isotype control (Dako). For Cleaved Caspase 3 staining, cells were incubated with 5% Normal Donkey Serum and anti-CD16/32 Fc block (Cone 2.4G2, UCSF Hybridoma Facility) for 15 minutes then stained with anti-Cleaved Caspase 3 (Asp175, Cell Signaling) or a rabbit IgG isotype control (Santa Cruz Biotechnology) for 45 minutes. For detection of the cleaved caspase-3, a PE donkey anti-rabbit secondary antibody (Jackson ImmunoResearch) was added for 20 minutes. For intracellular cytokine staining, cells were incubated with FITC-IFNγ (Clone XMG1.2), APC-IL-17 (Clone eBio17B7) and PE-IL-4 (Clone 11B11) or similarly conjugated isotype controls, all from eBioscience, for 30 minutes. Intracellular staining for Foxp3 was performed with an eBioscience PE anti-Foxp3 staining kit (Clone FJK-16s) following dead cell and surface staining. All samples were analyzed on the BD Biosciences LSR Fortessa or LSR II.

Western Blotting

Cells were pelleted, washed with PBS, and lysed in 1% NP-40 lysis buffer (150mM NaCl, 1mM EDTA, 50mM Tris-HCl, pH 7.6, 1mM NaVO₄, 1mM NaF, 1mM DTT, 1mM PMSF and Sigma protease inhibitor cocktail). Protein was quantified with the Bio-Rad DC Protein Assay. 50µg of protein was run out on 10% SDS-PAGE gels. Proteins were transferred to a nitrocellulose membrane and blocked with 5% bovine serum albumin in 1% Tween TBS. The membrane was probed with the following antibodies: mouse Bcl-2 (BD Pharmingen, Clone 3F11), human Bcl-2 (BD Pharmingen, Clone 6C8), or β-actin (Sigma).

In Vitro TCR Stimulation of Thymocytes and T Cells

Lymphoid organs were dissociated through 40µm strainers into RPMI media (L-glutamine, Sodium Pyruvate, Non-essential amino acids, Hepes, β-mercaptoethanol and Penicillin-Streptomycin). Thymocytes were plated at 2x10⁶ cells/well in triplicate in

96-well flat-bottom plates. Cells were left untreated or stimulated with either plate-bound anti-CD3 and anti-CD28 antibodies (clones 2C11 and PV-1, UCSF Hybridoma Facility) at the indicated concentrations for 18 hours. Death was assayed by Annexin V staining as described above. For assessment of cytokine production by splenic T cells, total splenocytes were stimulated for 4 hours with PMA and Ionomycin at the above concentrations in the presence of BD GolgiPlug per the manufacturer's protocol (Brefeldin A, BD Biosciences).

F5 and HY Negative Selection Assays

For F5 *in vivo* peptide injection studies, F5 TCR transgenic mice were injected intraperitoneally with 50nmol of Influenza NP₃₆₆₋₃₇₄ Strain A/NT/60/68 peptide (AnaSpec) in 200µl of PBS or PBS only for controls. A second injection was performed 24 hours after the first. Thymocytes were collected at 48 hours after the initial injection.

For F5 and HY thymic slice assays, tissue preparation was performed as previously described^{70,82}. In brief, thymic lobes were embedded in 4% GTG-NuSieve Low-melt Agarose (Lonza) in HBSS. 500µm slices were cut by Vibratome (1000 Plus, Leica) and placed in 0.4µm Cell Culture Inserts (BD Biosciences) in 6-well plates containing 1ml of RPMI media. TCR-specific (NP₃₆₆₋₃₇₄ Strain A/NT/60/68 and Smcy HY₇₃₈₋₇₄₆, AnaSpec) and control (NP₃₆₆₋₃₇₄ Strain A/PR/8/35 and OVA₂₅₇₋₂₆₄, AnaSpec) peptides were diluted to 100ng/ml in RPMI media and 1ml volume was added to the slices. Slices were incubated at 37°C in a plastic bag filled with 80% O₂+15% N₂+5% CO₂ (Blood Gas, Praxair) for 30 minutes. The peptide was then removed and slices were further incubated for the indicated times. Thymic slices were dissociated to create single cell suspensions and cell death was assessed by Cleaved Caspase 3 staining as described above.

Mixed Lymphocyte Reaction

Balb/c (allogeneic) and C57BL/6 (syngeneic) splenocytes were harvested as described above and subjected to 2000rads of γ-irradiation. Splenocytes were washed with RPMI media several times and plated at 1x10⁵ cells/well in a round-bottom 96-well plate. T cells (C57BL/6 responders) were purified from lymph nodes and spleen by negative selection columns (T Cell Enrichment Columns, R&D Systems) and labeled with 1µM Cell Trace CFSE (Invitrogen) according to the manufacturer's protocol. T cells were seeded on top of stimulators at 1x10⁵ cells/well in triplicate. Proliferation was assessed by CFSE dilution after 4 days.

Anti-Nuclear Antibody ELISA

Serum anti-nuclear antibodies were quantified using a Mouse Anti-Nuclear Antibody Total Ig kit (Alpha Diagnostic International) per the manufacturer's protocol. Briefly, samples were diluted at 1:100 in Low NSB Sample Diluent and added to a 96-well plate pre-coated with purified ENA. Quantification was performed relative to provided standards. The plate containing diluted sera and standards was incubated for 1 hour at room temperature. Anti-mouse Ig HRP was added for 30 minutes followed by TMB substrate for 15 minutes. The reaction was stopped and the plate was read at 450nm. Absorbance at 630nm was subtracted as background.

H&E Staining

Organs were fixed in formalin, embedded in paraffin, and sliced into 5µm sections by microtome. Sections were stained with Hematoxylin and Eosin (Fisher Scientific) by standard procedures. Images were captured with LAS Core V4.0 software (Leica) on a Leica DFC500 microscope equipped with a Leica DM2500 camera at 20X magnification (HCX PL-FLUOTAR 20X/0.50 objective, Leica).

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Figure 1

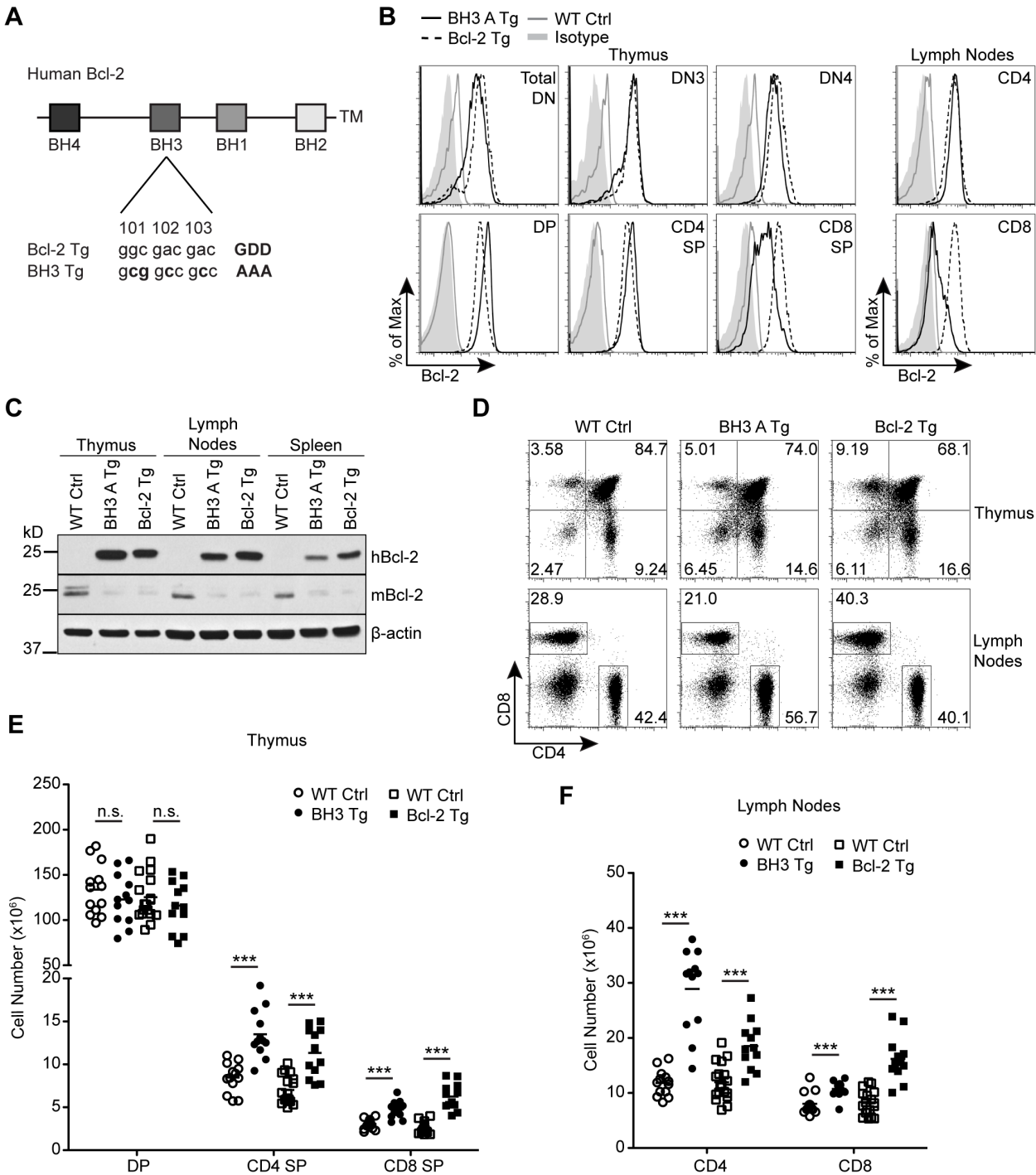


Figure 1. Generation of Bcl-2 BH3 domain mutant transgenic mice. (A) Diagram of the mutation made to the BH3 domain of human Bcl-2 to abolish BH3 domain function in the Bcl-2 BH3 mutant transgenic (BH3 Tg) mice. Amino acids 101-103 of human Bcl-2 were mutated from Glycine-Aspartate-Aspartate to Alanine-Alanine-Alanine to abolish BH3 domain function⁶⁴. (B) Flow cytometric analysis of intracellular Bcl-2 expression in BH3 Tg, line A (BH3 A Tg) versus *lck^{Pr}*-Bcl-2 (Bcl-2 Tg) and wild-type (WT) thymocyte and mature T cell populations. DN populations were gated based on CD25 and CD44 expression. (C) Western blot analysis comparing human versus mouse Bcl-2 expression in the thymus, lymph nodes and spleen of BH3 A Tg versus Bcl-2 Tg and WT mice. (D) Flow cytometric analysis of CD4 versus CD8 T cell populations in the thymus and lymph nodes. (E) DP, CD4 SP and CD8 SP thymocyte cell numbers in 6-week-old BH3 Tg and Bcl-2 Tg mice compared to WT littermate controls. (F) Lymph node mature CD4 and CD8 T cell numbers in 6-week-old BH3 Tg and Bcl-2 Tg mice compared to WT littermate controls. The transgenic mice in B-D were age-matched within one week and compared to a littermate non-transgenic WT control. All mice were between 6 and 10-weeks-old. B-F are representative of or compiled from at least 3 independent experiments. Statistics here and in the following figures were calculated by Student's t-test: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. not significant.

Figure 2

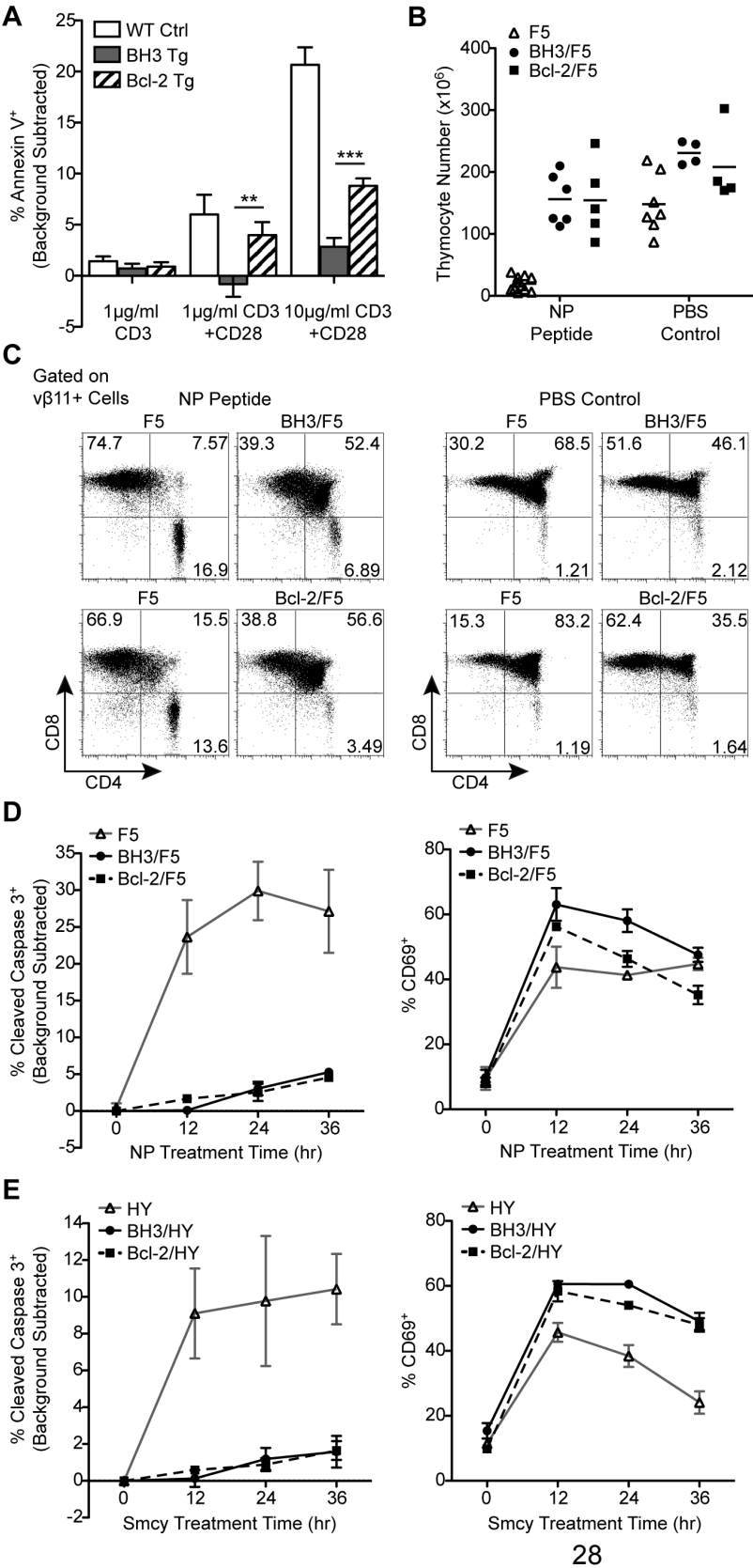


Figure 2. BH3 transgene expression efficiently blocks TCR-mediated thymocyte apoptosis *in vitro* and in F5 and HY TCR transgenic models of negative selection.

(A) Thymocytes were stimulated for 18 hours with anti-CD3 and anti-CD28 (20ug/ml) and analyzed for the percentage of Annexin V⁺ cells by flow cytometry. The percentage of Annexin V⁺ thymocytes in samples left untreated for 18 hours (i.e. background) was subtracted. (B) Total live thymocyte number in thymii collected 48 hours after injection with the F5 TCR-specific peptide, NP, or PBS. (C) CD4 versus CD8 flow cytometric analysis of F5 TCR⁺ (vβ11⁺) thymocytes from mice treated as in B. Data in B and C are representative of or pooled from at least 5 independent experiments per transgenic genotype. Mice were between 6 and 9-weeks-old and F5 control mice were littermates to BH3/F5 and Bcl-2/F5 mice. (D) Percentage of Cleaved Caspase 3⁺ thymocytes by flow cytometric analysis treated with 100ng/ml NP peptide for the indicated times. The percentage of Cleaved Caspase 3⁺ thymocytes treated with an irrelevant peptide (i.e. background) was subtracted at each time point. Efficient activation of thymocytes by the NP peptide was assessed by flow cytometric analysis of CD69 surface expression. Data are representative of 3 independent experiments. (E) Percentage of Cleaved Caspase 3⁺ thymocytes by flow cytometric analysis treated with 100ng/ml of the HY TCR-specific peptide, Smcy, for the indicated times. Background was subtracted as in D. Proper thymocyte activation was confirmed by analysis of CD69 expression. Data are representative of 2 independent experiments.

Figure 3

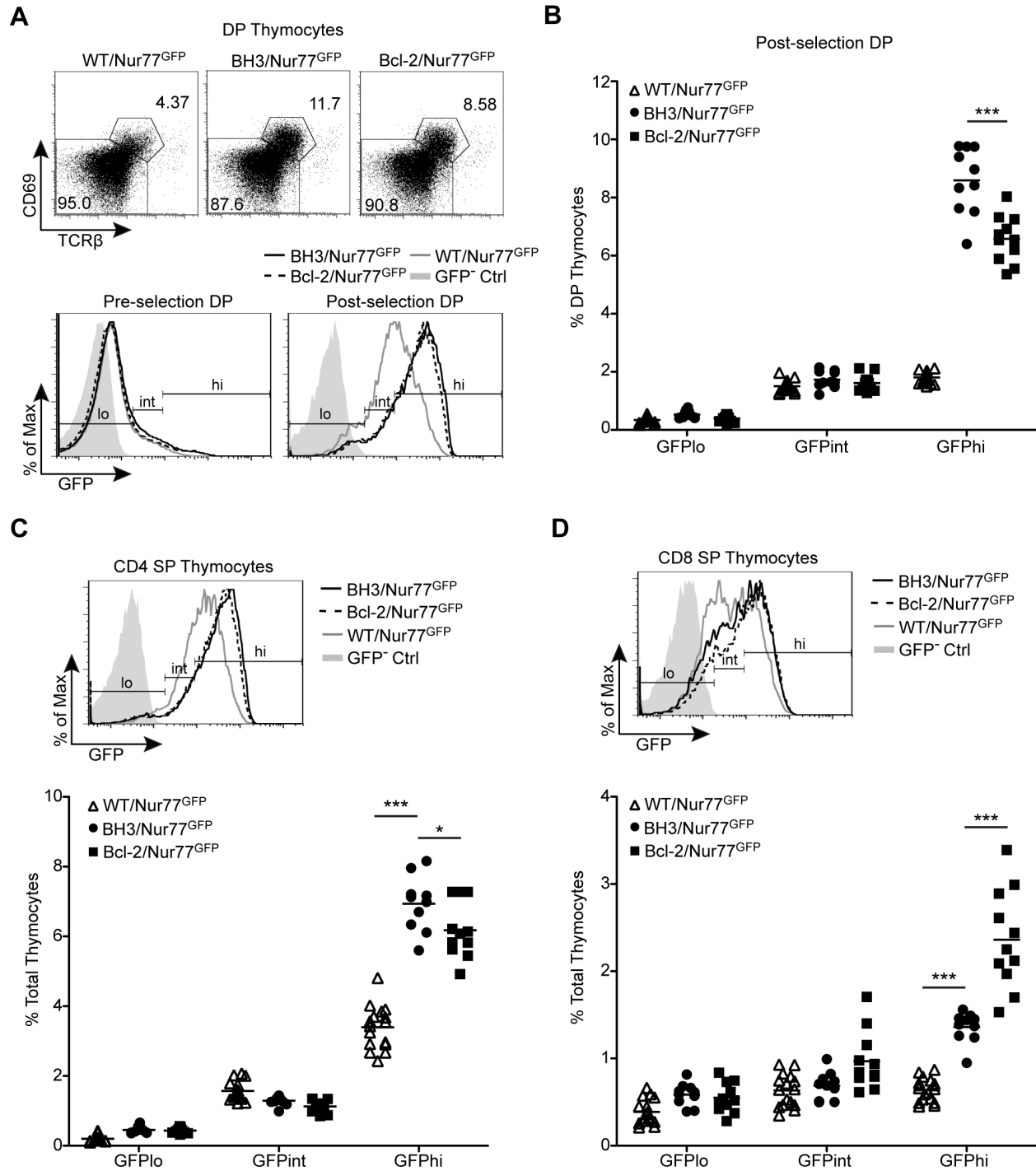


Figure 3. BH3 transgene expression rescues high affinity TCR clones in a polyclonal system (A) Flow cytometric analysis of GFP expression in pre-selection ($CD69^- TCR\beta^-$) versus post-selection ($CD69^+ TCR\beta^+$) DP thymocytes from Nur77^{GFP} mice expressing the BH3 and Bcl-2 transgenes. The WT/Nur77^{GFP} post-selection DP histogram was used to set the GFP low (lo), intermediate (int), and high (hi) gates. The left end of the “int” gate was set at the base of the GFP⁻ Ctrl histogram and right end was set at the mean GFP fluorescence intensity of the WT/Nur77^{GFP} histogram. The “lo” and “hi” gates were extended from the left and right ends of the “int” gate, respectively. (B) Percentage of DP thymocytes that were post-selection and GFP lo, int and hi as defined in A. (C and D) Flow cytometric analysis of GFP expression in CD4 SP (C) and CD8 SP (D) with the gates from post-selection DP cells in A applied. The GFP lo, int and hi percentages were quantified. All mice were 6-weeks-old and WT/Nur77^{GFP} and GFP⁻ Ctrl mice were littermates to the BH3/Nur77^{GFP} and Bcl-2/Nur77^{GFP} mice. Data were pooled from 10 independent experiments.

Figure 4

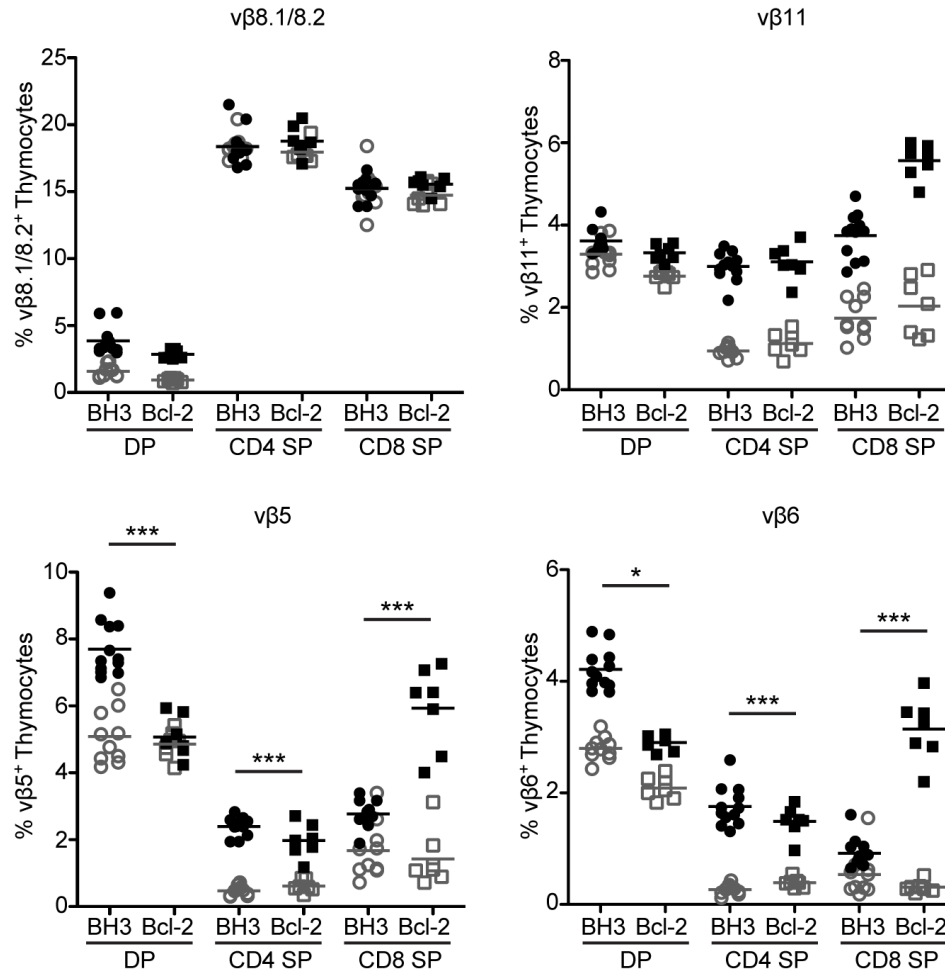


Figure 4. Superantigen negative selection is inhibited in BH3 Tg mice

Flow cytometric analysis of TCR vβ chain expression in DP, CD4 SP and CD8 SP thymocyte subsets from BH3 Tg and Bcl-2 Tg mice (closed shapes, black) compared to littermate wild-type controls (open shapes, grey) on a C57BL/6 X CBA/J genetic background. All mice were 8-weeks-old.

Figure 5

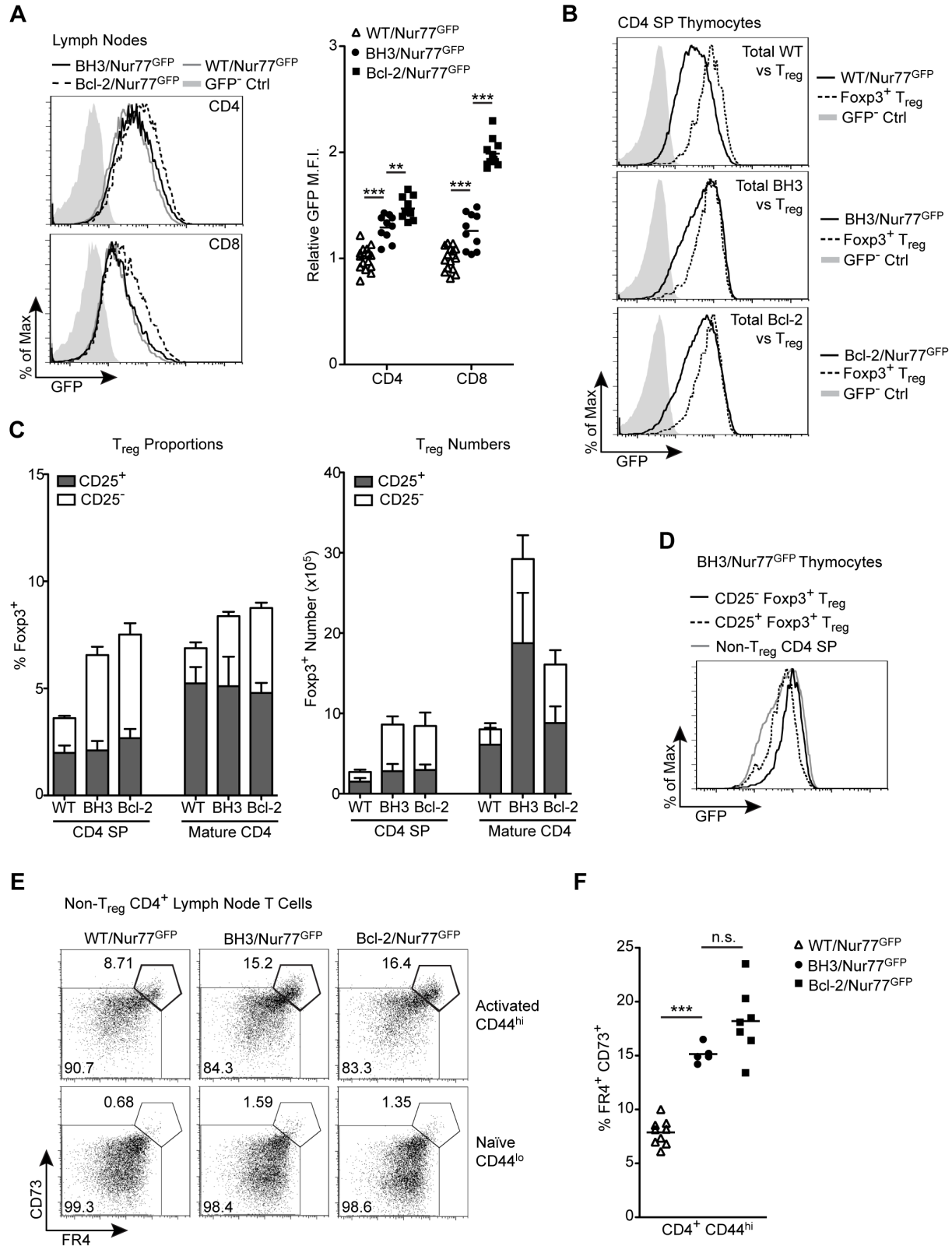


Figure 5. BH3 Tg autoreactive T cells are restrained by alternative tolerance mechanisms (A) Flow cytometric analysis of GFP expression by mature CD4 and CD8 lymph node T cells. Quantification indicates the fold change in GFP mean fluorescence intensity (M.F.I.) relative to WT/Nur77^{GFP} CD4 and CD8 samples. GFP M.F.I. was normalized between experiments by subtracting the M.F.I. of a GFP⁻ control. $n \geq 10$ per genotype. (B) Comparison of GFP expression by total CD4 SP thymocytes versus Foxp3⁺ CD4 SP T_{reg} cells. (C) Quantification of flow cytometric analysis of CD25⁺ versus CD25⁻ T_{reg} cells (CD4⁺ Foxp3⁺) in the thymus and lymph nodes. $n \geq 7$ per genotype. (D) Comparison of GFP expression levels in CD25⁺ versus CD25⁻ T_{reg} cells from BH3 Tg mice. (E) Flow cytometric analysis of non-T_{reg}, anergic CD4 T cells in the lymph nodes. T_{reg} cells were gated out by high expression of GITR and CD25. Anergic cells, indicated by the bolded gates, express high levels of CD44, FR4 and CD73. (F) Quantification of anergic CD4 T cells gated as described in E. Mice were 6-weeks-old in A-D and 7-weeks-old in E-F. All data are representative of or compiled from at least 3 independent experiments.

Figure 6

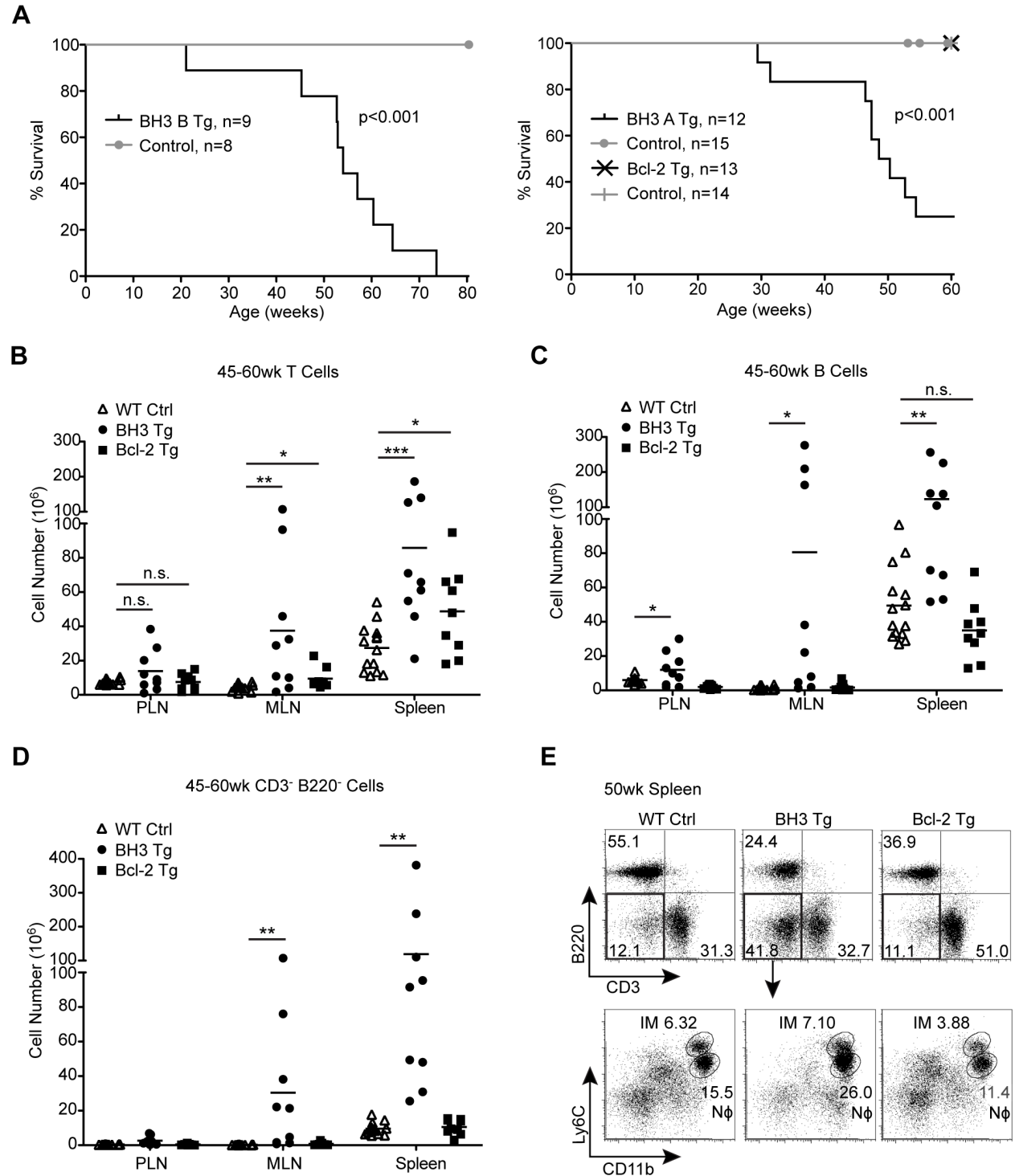


Figure 6. BH3 Tg mice have a shortened lifespan and exhibit lymphoid hyperplasia

(A) Kaplan-meyer curves for BH3 Tg lines A and B and Bcl-2 Tg mice compared to littermate WT controls. Statistical significance was calculated using the Log-rank test. (B-D) Quantification of CD3⁺ T cells (B), B220⁺ B cells (C) and CD3⁻ B220⁻ Myeloid/other cells (D) in the peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and spleen of 45 to 60-week-old mice. WT controls were littermates to BH3 Tg and Bcl-2 Tg mice. (E) Flow cytometric analysis of CD11b and Ly6C expression on CD3⁻ B220⁻ splenocytes from 50-week-old mice. Ly6C^{hi} CD11b⁺ inflammatory monocytes (IM) and Ly6C^{lo} CD11b⁺ neutrophils (N ϕ , also Ly6G⁺) are gated. Plots are representative of at least 5 mice per genotype.

Figure 7

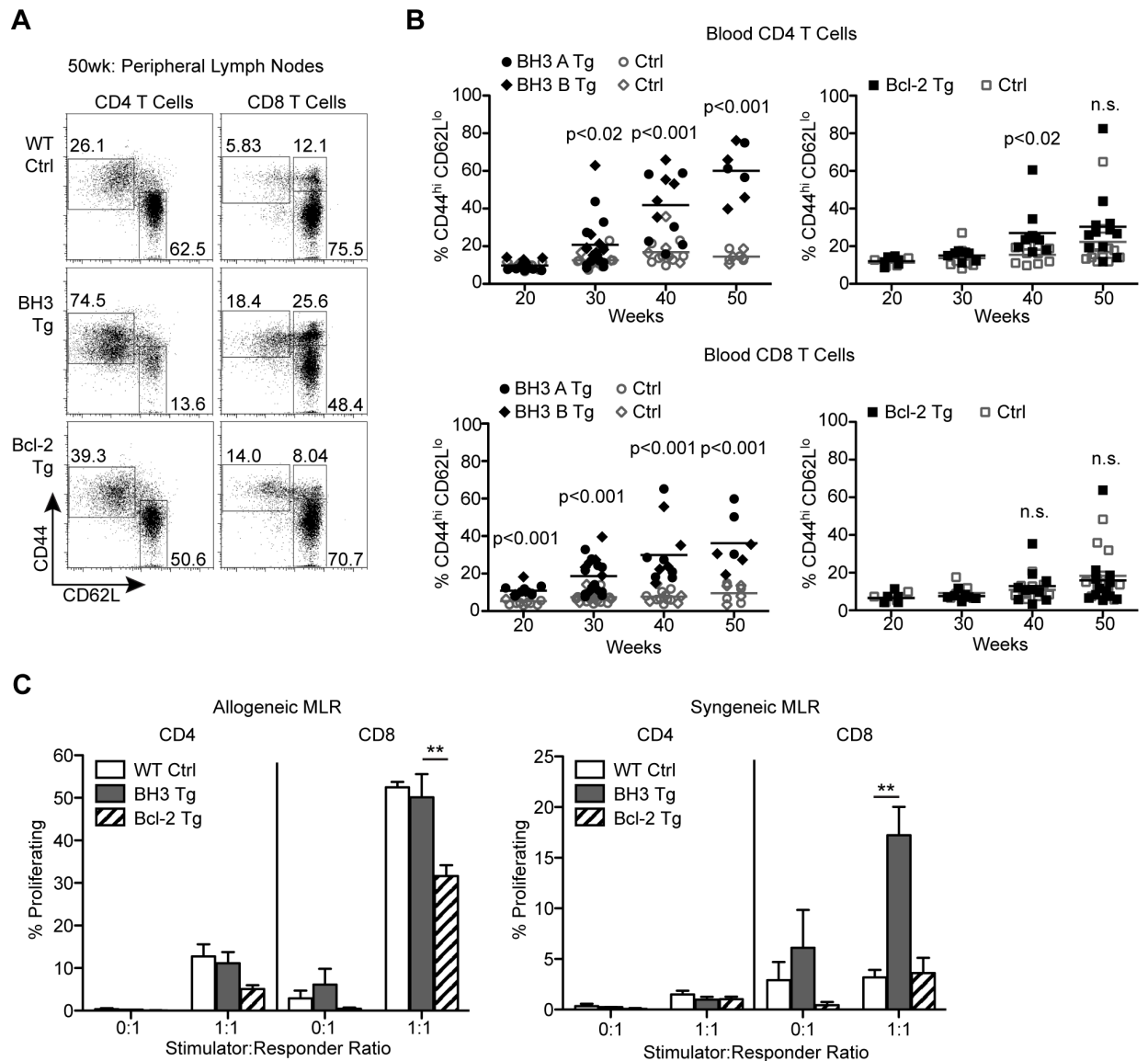


Figure 7. BH3 Tg mice accumulate activated, autoreactive T cells

(A) Flow cytometric analysis of activated CD4 and CD8 peripheral lymph node T cells by CD44 and CD62L expression in 50-week-old mice. For CD4 T cells, gates indicate activated (CD44^{hi} CD62L^{lo}) and naïve (CD44^{lo} CD62L^{hi}) populations. For CD8 T cells, activated effector memory (CD44^{hi} CD62L^{lo}) and activated central memory (CD44^{hi} CD62L^{hi}) versus naïve (CD44^{lo} CD62L^{hi}) populations are gated. (B) Compiled analyses of the percentage of activated T cells (CD44^{hi} CD62L^{lo}) in the peripheral blood of aged mice. Controls (Ctrl) are littermates to the Tg mice as denoted by data point shape. (C) Mixed lymphocyte reaction: purified “responder” T cells from 20-week-old mice were cultured with allogeneic (Balb/c) or syngeneic (C57BL/6) irradiated splenocyte “stimulators” at the indicated ratios. Proliferation was measured by CFSE dilution over 4 days. Data are representative of 3 independent experiments.

Figure 8

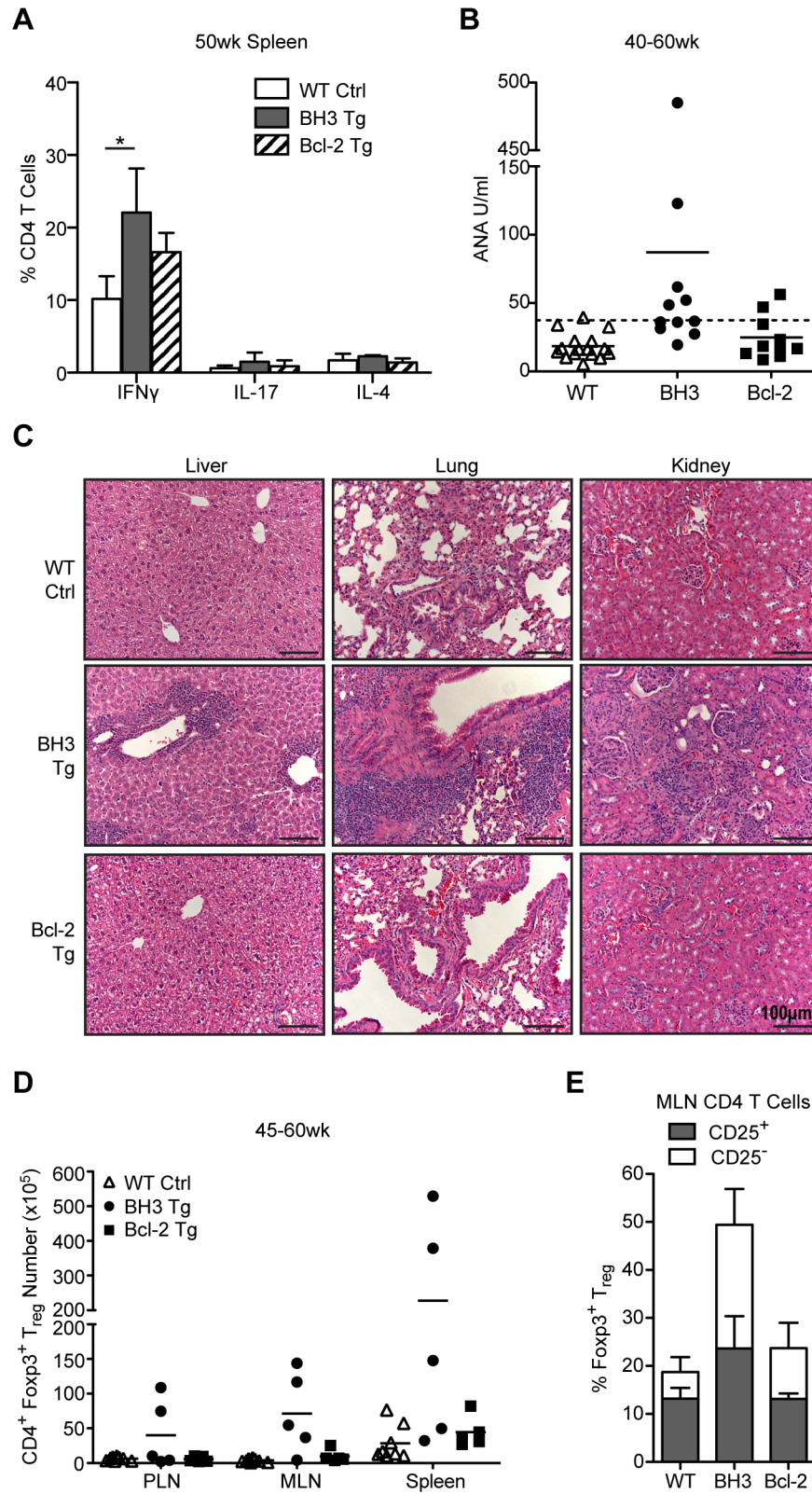


Figure 8. T cell-mediated autoimmune pathology is apparent in BH3 Tg mice

(A) Quantification of flow cytometric analysis of T helper cell cytokine expression. Splenic CD4 T cells from 50-week-old mice were stimulated with PMA and Ionomycin for 4 hours in the presence of brefeldin A to allow cytokine accumulation. $n \geq 3$ mice per genotype. (B) Serum anti-nuclear antibody (ANA) quantification by ELISA. Dashed line represents two standard deviations above the WT mean. Samples above this line are considered positive for ANA. (C) Hematoxylin and Eosin staining of liver, lung and kidney sections from 50-week-old mice. Images were captured at 20X magnification. Data are representative of at least 3 mice per genotype. (D) Quantification of CD4⁺ Foxp3⁺ T_{reg} cells in the lymphoid organs of aged mice. (E) Percentage of CD4 T cells that are T_{reg} cells (CD25⁺ versus CD25⁻) in the mesenteric lymph nodes of 45 to 60-week-old mice. $n \geq 5$ mice per genotype.

Chapter 3

The regulation of Nor-1 and Nur77 mitochondrial translocation during thymocyte apoptosis

Summary

The Nr4a nuclear receptors Nor-1 and Nur77 play an important role in inducing apoptosis of highly self-reactive thymocytes during T cell development. In response to strong TCR engagement, Nor-1 and Nur77 translocate from the nucleus to the mitochondria where they promote apoptosis through conversion of Bcl-2 into a pro-apoptotic effector molecule. Protein Kinase C (PKC) signaling is necessary for inducing the translocation of Nor-1 and Nur77 to the mitochondria, however the mechanism downstream of PKC remains undefined. Here, taking a bottom-up approach, we sought to identify the residues of Nor-1 critical for mitochondrial translocation by analyzing the localization of Nor-1 deletion mutants in a CD4⁺ CD8⁺ thymocyte-like cell line, 16610D9. Using a subcellular fraction procedure, we demonstrate that deletion of the N-terminal transactivation domain of Nor-1 prevents TCR-mediated translocation from the nucleus to the mitochondria. Within this domain, deletion of residues 1-82 improves translocation, excluding a role for these residues in directly promoting translocation. Surprisingly, three regions spanning the remainder of the transactivation domain are independently sufficient for translocation: residues 83-146, 147-206 and 207-290. Further dissection of these regions will elucidate the residues critical for translocation and allow delineation of upstream regulation.

Introduction

The stochastic process of T cell receptor (TCR) gene rearrangement generates a T cell repertoire with an astounding number of antigen specificities, thus arming the immune system with the ability to recognize an almost infinite array of potential pathogens⁸³. However, a dangerous consequence of the random nature of this process is the generation of T cells that are reactive to self. To safeguard against autoimmunity, developing T cells with high affinity for self-peptide are eliminated in thymus through a process termed negative selection, ensuring production of a largely self-tolerant mature T cell repertoire⁷.

Negative selection occurs starting at the CD4 CD8 double positive (DP) stage of T cell development in the thymic cortex¹⁰. DP thymocytes are acutely susceptible to apoptosis and have a maximum lifespan of about 3 days⁸³. Most DP cells undergo apoptosis due to a lack of TCR stimulus, referred to as death by neglect. Those with high TCR reactivity to ubiquitously expressed self-antigens are signaled to die by negative selection. A small percentage of DP thymocytes mature into CD4 or CD8 single positive (SP) thymocytes and migrate into the thymic medulla. Here, SP thymocytes with high affinity for tissue-restricted antigens expressed by medullary epithelial cells under the control of the autoimmune regulator (AIRE) gene are subjected to negative selection¹⁰. Deficiency in AIRE results in the development of a T cell-mediated, multi-organ autoimmune disease due to impaired deletion of these SP cells³⁶. Defective mitochondrial apoptotic machinery leads to similar disease pathology, as observed in mice doubly deficient in pro-apoptotic Bcl-2 family members Bim and Puma⁴⁴.

In addition to pro-apoptotic Bcl-2 family members, the Nr4a family of orphan nuclear steroid receptors is thought to play an important role in promoting apoptotic

deletion of negatively selected thymocytes³². The family consists of three members, Nur77, Nor-1 and Nurr1, that share homologous transactivation, DNA binding and ligand binding domains. Their activity is thought to be ligand-independent, as they are structurally locked in a constitutively active conformation⁸⁴. T cell-specific over-expression of Nur77 or Nor-1 in mice results in massive apoptosis of thymocytes^{16,17}. In contrast, expression of a dominant negative Nur77 protein that blocks the activity of all family members significantly protects thymocytes from death in F5 and HY TCR transgenic models of negative selection^{16,18}. Nur77^{-/-} mice have a partial defect in thymocyte apoptosis in OTII and BDC2.5 TCR transgenic models, suggesting redundancy between family members¹⁹. Strikingly, deficiency in all three family members results in a severe defect in T regulatory (T_{reg}) cell development and rapid onset of severe autoimmune pathology due to defective Foxp3 expression²¹. Hence, the Nr4a family may act to uphold T cell tolerance by both inducing thymocyte apoptosis and promoting T_{reg} cell development.

The mechanism by which the Nr4a family promotes apoptosis during negative selection has long been under investigation. As transcription factors, the Nr4a proteins target genes involved in apoptotic signaling through the death receptor pathway, including TRAIL and Fas ligand²³. Over-expression of a Nur77 mutant with increased transcriptional activity results in much greater apoptosis of thymocytes compared to a mutant lacking most of its transcriptional capacity²². However, expression of a dominant negative FADD protein, able to block signaling from multiple death receptors, does not result in a defect in negative selection¹⁴. Alternatively, the Nr4a family might promote apoptosis primarily through the mitochondrial death pathway. Reminiscent of an apoptotic mechanism described in cancer cells²⁹, we and others have shown that in response to strong TCR signals, Nur77 and Nor-1 can translocate from the nucleus to the mitochondria where they interact with anti-apoptotic protein Bcl-2^{26,27}. Bcl-2 is then converted from an anti-apoptotic protein to a pro-apoptotic protein through exposure of its BH3 domain, the “killer” domain of the Bcl-2 protein family. Inhibition of nuclear export by Leptomycin B prevents Bcl-2 BH3 domain exposure and significantly rescues thymocytes from apoptosis. Bcl-2 BH3 domain exposure also occurs in F5 and HY TCR transgenic thymocytes undergoing negative selection²⁶. Mitochondrial translocation and Bcl-2 BH3 domain exposure were demonstrated to be dependent on Protein Kinase C (PKC) signaling, however the mechanism downstream of PKC signaling remains ill-defined²⁵. As Nur77 and Nor-1 exist in a highly phosphorylated state at the mitochondria, it is plausible that translocation is mediated by a phosphorylation event downstream of PKC.

To begin to elucidate the downstream mechanism regulating Nur77 and Nor-1 mitochondrial translocation, we sought to identify regions of the proteins necessary for localization to the mitochondria. We demonstrate that several regions within the highly phosphorylated N-terminal transactivation domain are involved in regulating mitochondrial translocation. These data will guide future work to pinpoint the critical residues, allowing delineation of the upstream, PKC-dependent signaling pathways involved. Additionally, manipulation of nuclear versus mitochondrial localization through mutation of these residues could more conclusively demonstrate the relative importance of Nur77 and Nor-1's nuclear versus mitochondrial roles in promoting thymocyte apoptosis.

Results

To determine the regions of the Nor-1 protein necessary for translocation to the mitochondria, we assessed the subcellular localization of Nor-1 mutant proteins in a DP thymocyte-like cell line, 16610D9. Initially, large truncations were made at the boundaries between the conserved transactivation, DNA binding and ligand binding domains of the protein (Figures 1A-B). The nuclear localization sequences (NLS) of the Nor-1 protein are located within the DNA binding domain. To ensure proper localization to the nucleus following protein translation, NLS sequences were added to the N-terminus of mutants lacking the DNA binding domain (i.e. T NLS, Figure 1B). All mutants were N-terminally Flag tagged to facilitate detection by western blot.

Initially, nuclear versus cytoplasmic localization was assessed, due to the technical difficulty of isolating a pure mitochondrial fraction. Localization was assessed at steady-state (untreated) and in response to TCR-stimulation (PMA/Ionomycin), which should induce Nor-1 translocation. As shown in Figure 1C, TCR-stimulation only modestly induced translocation of the full-length Nor-1 protein (FL), reflected by an increased ratio of cytoplasmic to nuclear protein. In fact, a significant amount of protein was seen in the cytoplasmic fraction without stimulation. This may be due to protein mislocalization resulting from high levels of over-expression. Alternatively, as a cancer cell line, 16610D9 cells might have high steady-state levels of PKC activation, causing some translocation to occur in the absence of exogenous stimuli. Despite this phenomenon, we clearly see differential localization of the various Nor-1 mutants in Figure 1C. Surprisingly, a mutant lacking the ligand binding domain, Δ LB, behaves much like FL, despite the fact that Nor-1's putative Bcl-2 binding site is located within this domain (Figures 1A and 1C). Interestingly, a mutant lacking the transactivation domain, Δ T, was almost entirely localized to the nucleus while transactivation domain-only mutants T and T NLS were found exclusively in the cytoplasm (Figure 1C). This observation suggests that the transactivation domain is necessary for nuclear export and/or cytoplasmic localization.

To investigate whether the transactivation domain is also necessary for mitochondrial localization, we developed an optimized subcellular fractionation procedure to isolate a pure mitochondrial fraction from 16610D9 cells (see Methods). Consistent with our previous observation, Δ T was again localized primarily to the nucleus while T NLS was found only at mitochondria, under both untreated and PMA/Ionomycin-stimulated conditions (Figure 2). Also of note, greater localization of FL and Δ LB proteins to the mitochondria was evident with PMA/Ionomycin treatment compared to no treatment in this experiment, validating the ability of TCR-stimulation to induce translocation in 16610D9s. However, the purity of the mitochondrial fraction in the PMA/Ionomycin treated samples was questionable.

Serine 354 of Nur77, located within the A-box of the DNA binding domain, has previously been implicated in regulating Nur77 translocation in the T cell hybridoma, DO11.10²⁷. However, in 16610D9 cells, which more closely resemble DP thymocytes, we found that serine 354 does not play a role²⁵. To directly rule out involvement of the homologous residue of Nor-1, serine 377, we point mutated it to alanine and examined localization of the mutated protein. We found that mutation of serine 377 does not

prevent Nor-1 localization to the mitochondria, in agreement with our observation for Nur77 (Figure 2). In addition, we created two additional constructs with point mutations within the A-box that should abolish Nor-1 DNA binding activity (L375A) or DNA binding activity plus one of two nuclear localization signals (R371-4A). Interestingly, both mutants were able to localize to the mitochondria and mitochondrial targeting was enhanced with PMA/Ionomycin treatment (Figure 2). This suggests that regulation at the level of Nor-1 nuclear export might play a key role in controlling localization of Nor-1 to the mitochondria. Additionally, intact DNA binding activity might have an important function in keeping Nor-1 localized to the nucleus. ΔT restriction to the nucleus might therefore result from an inability to leave the nucleus and/or lost capacity to localize to the mitochondria.

To determine the region(s) within the transactivation domain critical for Nor-1 mitochondrial localization, we created serially truncated forms of T NLS (Figure 3A). Interestingly, all T NLS mutants were found primarily at the mitochondria, even under steady-state conditions (Figure 3B, note that 1-257 was not expressed). Mutant T NLS 1-148 was absent from the mitochondria under stimulated conditions, however this was likely the result of low expression levels. All mutants had residues 83-148 in common, suggesting that this region could be responsible for mitochondrial targeting of Nor-1.

To test this, we created a T NLS mutant with residues 82-206 deleted (T NLS $\Delta 82-206$, Figure 4A). Residues 148-206 were included in the deletion due to the somewhat ambiguous data for the T NLS 1-148 mutant in Figure 3B. These same residues were also deleted from the full-length version of the protein to create FL $\Delta 82-206$ (Figure 4A). To further dissect the 82-206 amino acid region, three N-terminal truncations of full-length Nor-1 were also made: FL -82, FL -146 and FL -206. Surprisingly, T NLS $\Delta 82-206$ was able to localize to the mitochondria, although expression levels were very low (Figure 4B). The purity of the mitochondrial fraction in this experiment was also poor and results should be weighed accordingly. However, consistent with the observation for T NLS $\Delta 82-206$, FL $\Delta 82-206$ and all of the N-terminal FL truncation mutants were able to translocate as well (Figure 4B). In fact, deletion of the first 82 amino acids might even enhance mitochondrial localization over the FL protein. In conflict with the T NLS mutant data (Figure 3B), these results indicate that residues 1-206 of Nor-1 are not necessary for mitochondrial localization. This suggests that more than one region within the transactivation domain might be sufficient for Nor-1 localization to the mitochondria.

Since FL -206 can localize to the mitochondria, but ΔT cannot, a region within residues 207-290 might be important for regulating mitochondrial localization. To test this, we deleted 207-290 from full-length Nor-1 (FL $\Delta 207-290$, Figure 5A) and compared the localization of this mutant with FL -206. In this experiment, the purity of the mitochondrial fraction was much improved, however construct expression levels were quite variable, making it more difficult to interpret relative translocation abilities. Consistent with previous experiments, however, ΔT and T NLS were for the most part reciprocally localized (Figure 5B). FL -206 was able to translocate to the mitochondria very well, seemingly much better than FL. The enhanced translocation ability of FL -206 could indicate that there is a region within residues 1-206 that inhibits translocation. Our findings above (Figure 4) suggest that this region could potentially be narrowed down to the first 82 amino acids. FL $\Delta 207-290$ was still able to translocate, with comparable

translocation ability to FL. This suggests that residues 207-290 are not necessary for translocation. However, the fact that FL -206 can translocate, while ΔT cannot, still strongly argues for 207-290 being sufficient for promoting translocation.

Our results thus far suggest that more than one region within the N-terminus is involved in promoting translocation, likely between residues 83 and 290, while at least one region acts to inhibit it, probably within the first 82 amino acids. To pinpoint the residues between 83 and 290 that are involved in promoting translocation, we created four additional mutants with various deletions within this region. Amino acids 1-82 were also deleted from all of the new mutants to relieve any direct inhibition on translocation (Figure 6A). In this experiment, fraction purity was improved and translocation was highly inducible with PMA/Ionomycin treatment. Interestingly, deletion of residues 1-82 in addition to 207-290 (FL -82 $\Delta 207-290$) dramatically improved translocation compared to the $\Delta 207-290$ mutant (Figure 6B). This confirms that residues 207-290 are not necessary for translocation and also further supports the existence of an inhibitory region within residues 1-82. Interestingly, all remaining mutants in this group were able to localize to the mitochondria, altogether suggesting that regions 83-146, 147-206 and 207-290 are each independently sufficient for promoting translocation (Figures 6A-B).

To further narrow down the residues necessary for Nor-1 translocation within these three regions, we performed a bioinformatic phosphoproteomic analysis using two algorithms, Phosida and DisPhos1.3, to identify serine and threonine residues with the greatest likelihood of being phosphorylated. To increase stringency, we also excluded residues that were not well conserved between mammals and lower organisms, down to zebrafish. This analysis identified 5 conserved residues within 83-146, 1 within 147-190 and 4 within 207-290. Truncation and point mutant constructs were created to test the importance of these residues for translocation (Figure 7). All mutants depicted were successfully cloned except FL $\Delta 147-290$ [S133A, S139A, S142A, T143A, T146A]. Expression of mutants -82 $\Delta 132-290$, -131 $\Delta 147-290$, -206 $\Delta 269-290$ have been confirmed, however the localization of all of these mutants awaits testing.

To begin to compare the regulation of Nor-1 versus Nur77 translocation, several Nur77 truncation mutants were created guided by our observations made for Nor-1 (Figure 8A). Interestingly, some mutants were undetectable by Flag immunoblot, but visible by immunoblot with a Nur77 polyclonal antibody. All but the -205 mutant were ultimately detectable and it possible that the -205 mutant was not expressed (Figure 8B). Similar to Nor-1 ΔT , Nur77 ΔT was found primarily in the nucleus and did not localize to the mitochondria, even with TCR stimulation. This suggests that the transactivation domain is also important for Nur77 translocation. However, ΔLB was also absent from the mitochondria, but it is possible that this was due to low expression levels. Interestingly, deletion of the DNA binding domain (ΔDB) resulted in greater localization to the mitochondria despite addition of NLS sequences, consistent with the Nor-1 L375A and R371-4A mutant data. This could suggest that DNA binding is important for keeping Nur77 in the nucleus. Deletion of residues 1-115 resulted in greater mitochondrial localization, which could indicate that Nur77 also has an inhibitory region within the most N-terminal region of the transactivation domain.

Discussion

Nur77 and Nor-1 have long been thought to play a critical role in promoting apoptosis during thymocyte negative selection. However, the mechanism by which they promote apoptosis remains ill-defined. In response to strong TCR stimuli, Nur77 and Nor-1 translocate to the mitochondria, where they are thought to promote death by conversion of Bcl-2 into a pro-apoptotic molecule. PKC signaling is necessary for promoting translocation, but the signals downstream of PKC necessary for translocation have yet to be elucidated. In this study, we identified three regions within the Nor-1 transactivation domain that are independently sufficient for Nor-1 localization to the mitochondria. Additionally, one region within the first 82 amino acids of the protein appears to inhibit translocation. Consistent with our observations, removal of the N-terminus of Nur77 has previously been shown to block translocation in other cell types, including LNCaP prostate cancer, H460 lung cancer, and PC12 adrenal gland tumor cells^{85,86,87,88}.

As Nur77 and Nor-1 are highly phosphorylated at the mitochondria, phosphorylation events downstream of PKC within the N-terminus might facilitate mitochondrial translocation. Indeed, several MAP kinases have been implicated in regulating translocation through phosphorylation of Nur77's N-terminus in LNCaP, H460 and PC12 cells^{85,86,87,88}. However, a panel inhibitor study performed by our lab demonstrated that inhibition of JNK, Erk1/2 and p38 MAP kinases as well as Akt does not affect mitochondrial translocation of Nur77 or Nor-1 in thymocytes²⁵. Only inhibition of PKC was able to significantly block translocation, though it is unlikely that PKC phosphorylates Nur77/Nor-1 directly as no PKC consensus sites are present in the N-terminus. It is possible, however, that multiple kinases function redundantly downstream of PKC to promote translocation. Our observation that multiple regions within the transactivation domain are sufficient to promote translocation lends some support to this hypothesis.

It is unclear what allows Nur77 and Nor-1 to localize to the mitochondria once out of the nucleus. No conserved mitochondrial targeting sequence is present in either protein. Interestingly, the transcription factor p53 similarly lacks a mitochondrial targeting sequence, but can also localize to the mitochondria. p53 may reside at the outer mitochondria membrane due to its interactions with Bcl-2 family proteins⁸⁹. Similarly, Bcl-2 binding might facilitate Nur77/Nor-1 localization to the mitochondria^{26,29}. However, we show here that deletion of Nor-1's ligand binding domain, containing its putative Bcl-2 binding site, does not prevent localization of Nor-1 to the mitochondria. Interestingly, the Nor-1 nuclear export signals are also located within this domain and therefore are seemingly unnecessary for translocation. It has previously been suggested that Nur77 heterodimerization with RXR α facilitates Nur77 nuclear export by way of RXR α 's nuclear export signal. Nur77 is thought to interact with RXR α through its DNA binding domain, inhibiting Nur77 DNA binding activity and activating the nuclear export signal of RXR α ⁹⁰. Our finding that the Nor-1 DNA binding domain is also unnecessary for Nor-1 mitochondrial localization argues against this mechanism. Interpretation of our results, however, hinges on the effectiveness of our exogenously

added NLSs. It is possible that our T NLS mutant is not well targeted to the nucleus and can therefore preferentially localize to the mitochondria.

Our finding that three regions within the N-terminal transactivation domain are sufficient for mitochondrial targeting raises some suspicion, as it seems highly unlikely that three independent regions could directly promote interaction with the mitochondria. It seems more likely that there is a region outside of the transactivation domain, common between all mutants containing residues 291-628, that is responsible for interaction with the mitochondria, while the transactivation domain regions just facilitate translocation. It is also possible that the specific transactivation domain regions that we identified are not necessary for inducing translocation, but that a certain number of residues must exist N-terminally of ΔT in order to achieve an appropriate conformation for nuclear export/mitochondrial targeting. However, these hypotheses cannot explain the localization of all of the T NLS mutants (Figure 3B) to the mitochondria. This observation suggests that either amino acids 83-148 are capable of facilitating interaction with the mitochondria or that localization of the T NLS mutants to the mitochondria is an artifact of our system. While our results give us confidence in the purity of the nuclear versus cytoplasmic fractions, it is difficult to be sure of the purity of the mitochondrial fraction. It is possible that other organelles, such as the endoplasmic reticulum, are being isolated with the mitochondria. Cytoplasmic contamination could also be an issue. Going forward, analysis of additional mutants should further validate the integrity of our system.

Identification of the residues driving translocation will not only allow us to elucidate upstream regulation, but also to examine the relative importance of Nor-1's mitochondrial versus reputed nuclear killing abilities. Nor-1 mutants that do not localize to the mitochondria or that constitutively localize to the mitochondria could be created, with and without the ability to bind DNA. Here, we identified a mutation that could be potentially used to abolish DNA binding activity, L375A. We found that this mutant was able to localize to the nucleus, but translocated to the mitochondria better than FL in response to TCR-stimulus. Intriguingly, assessment of transduction efficiency of this construct in comparison to FL, S377A and R371-4A hints at an important nuclear killing function for Nor-1. FL and S377A yield low percentages of GFP⁺ cells post-transduction, potentially because over-expression of these constructs causes apoptosis of many of the transduced cells. Conversely, L375A and R371-4A yield high percentages of GFP⁺ cells (approximately 3-fold higher), suggesting that these proteins cause less apoptosis (data not shown). Future studies should explore the relative contribution of nuclear and mitochondrial killing pathways both in 16610D9s and primary thymocytes, if feasible, as our findings in 16610D9s may not reflect what is happening in untransformed, normal cells *in vivo*.

Materials and Methods

Generation of Nor-1 mutant constructs

Nor-1 mutants were generated by PCR amplification from mouse Nor-1 cDNA (Addgene) (or subsequently generated constructs) using Biorad iProof High-Fidelity

DNA Polymerase. Primers are listed in Table 1. Deletion mutants were created using a standard PCR site-directed mutagenesis procedure and inside and outside primers are specified in Table 1. Nor-1 point mutants were created using standard site-directed mutagenesis or a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Final PCR products were cloned into an MSCV-ires-GFP vector (generously provided by Lin He) at EcoRI and/or BglII restriction sites.

16610D9 transduction

Phoenix cells (at 80-90% confluence) were transfected with 3 μ g MSCV, 0.5 μ g VSV-G and 1 μ g gag-pol helper plasmid (Nolan lab) with 10 μ l Lipofectamine 2000 (Invitrogen) per well of a 6-well plate in 1ml Opti-MEM (Invitrogen) media supplemented with 10% fetal bovine serum (FBS). Five hours post-transfection, the media was changed to 2ml Opti-MEM supplemented with 10% FBS, penicillin/streptomycin and β -mercaptoethanol (16610D9 media). Two days post-transfection, the viral supernatant was syringe filtered (0.2 μ m) and supplemented with 4 μ g/ml Polybrene (Santa Cruz Biotechnology) and added to 2-4 x 10⁶ 16610D9 cells in a 6-well format. The 16610D9 cells were spun at 2500rpm for 1 hour at room temperature and cultured for 8 hours. The media was changed to fresh 16610D9 media and the cells were cultured for 2 days. Successful transduction was confirmed by analysis of GFP expression by flow cytometry prior to subcellular fractionation.

Nuclear/cytoplasmic subcellular fractionation

Retrovirally transduced 16610D9 cells were TCR-stimulated with 60ng/ml PMA (Calbiochem) and 3 μ M Ionomycin (Sigma) for 24 hours. 2 x 10⁷ stimulated D9 cells were collected for fractionation and washed once with PBS. The cell pellet was then resuspended in 200 μ l of Solution A (10mM Hepes-KOH pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.5% NP-40, protease and phosphatase inhibitors) and incubated on ice for 10 minutes. The suspension was centrifuged at 3300g for 5 seconds to pellet the nuclei. The cytoplasmic supernatant was transferred to a new tube and cleared of nuclear debris by centrifugation at 1000g for 5 minutes two times. The nuclear pellet was washed 3 times with PBS with centrifugation for 5 minutes at 14000g between each wash. The nuclear pellet was lysed by adding 50 μ l of Solution B, rack whacking 10-12 times and incubating on ice for 20 minutes. Debris was cleared by centrifugation at 14000g for 1.5 minutes and the pure nuclear supernatant was transferred to a new tube.

Nuclear/mitochondrial/cytoplasmic subcellular fractionation

Retrovirally transduced 16610D9 cells were TCR-stimulated as described above. 2 x 10⁷ stimulated D9 cells were collected for fractionation and washed once with PBS. The cell pellet was then gently resuspended in 200 μ l of Hypotonic Buffer (20mM Hepes pH 7.4, 10mM KCl, 1mM EGTA, 1mM EDTA, protease and phosphatase inhibitors) and incubated on ice for 20 minutes. The suspension was then transferred to a 2ml glass dounce homogenizer and dounced 10 times, avoiding bubbles below the pestle. The homogenate was transferred to an eppendorf tube and 50 μ l of Isotonic Buffer (20mM Hepes pH 7.4, 10mM KCl, 7.5mM MgCl₂, 1mM EGTA, 1mM EDTA, 1.25M sucrose,

protease and phosphatase inhibitors) was added with gentle resuspension. The mixture was immediately spun down for 5 seconds at 3300g to pellet the nuclei. The supernatant was then removed and transferred to a new tube and cleared of nuclear debris by centrifugation at 1000g for 5 minutes two times. The supernatant was then spun at 10000g for 30 minutes to pellet the mitochondria. The supernatant was transferred to Beckman ultracentrifuge tubes (#343775) and spun in a TLA-100 rotor at 100000g for 30 minutes to obtain a pure cytoplasmic fraction. The mitochondrial pellet was thoroughly resuspended in 50 μ l of Mitochondrial Lysis Buffer (10mM Tris pH 7.5, 5mM EDTA, 150mM NaCl, 1% NaCl, 1% Triton-X 100) and incubated on ice for 30 minutes. Mitochondrial lysates were cleared by centrifugation at 14000g for 15 minutes. The nuclear pellet was washed three times with 250 μ l of Nuclear Wash Buffer (10mM Tris pH 7.5, 20mM KCl, 1.5mM MgCl₂, 250mM sucrose, 0.5% Triton-X 100) with 5 second spins between washes at 3300g. The nuclear pellet was lysed by adding 50 μ l Nuclear Lysis Buffer (10mM Tris pH 7.5, 1.5mM MgCl₂, 400mM NaCl, 1mM EDTA, 25% glycerol, 1% Triton-X 100) and rack whacking 15 times and incubating on ice for 30 minutes. Nuclear lysates were cleared by centrifugation at 14000g for 15 minutes.

Western blot

Protein fractions were quantified using the Bio-Rad *DC* Protein Assay according to the manufacturer's protocol. Equivalent amounts of each fraction (generally between 20-40 μ g) were run on 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membrane and blocked with 5% bovine serum albumin in 1% Tween TBS. Membranes were probed with the following antibodies: Flag (Sigma, F3165), Hsp60 (Santa Cruz Biotechnology, sc-13115 or BD Pharmingen, 611562), Lamin B1 (Abcam, ab16048), β -actin (Abcam, ab6276), Nur77 (Santa Cruz Biotechnology, sc-5569).

Figure 1

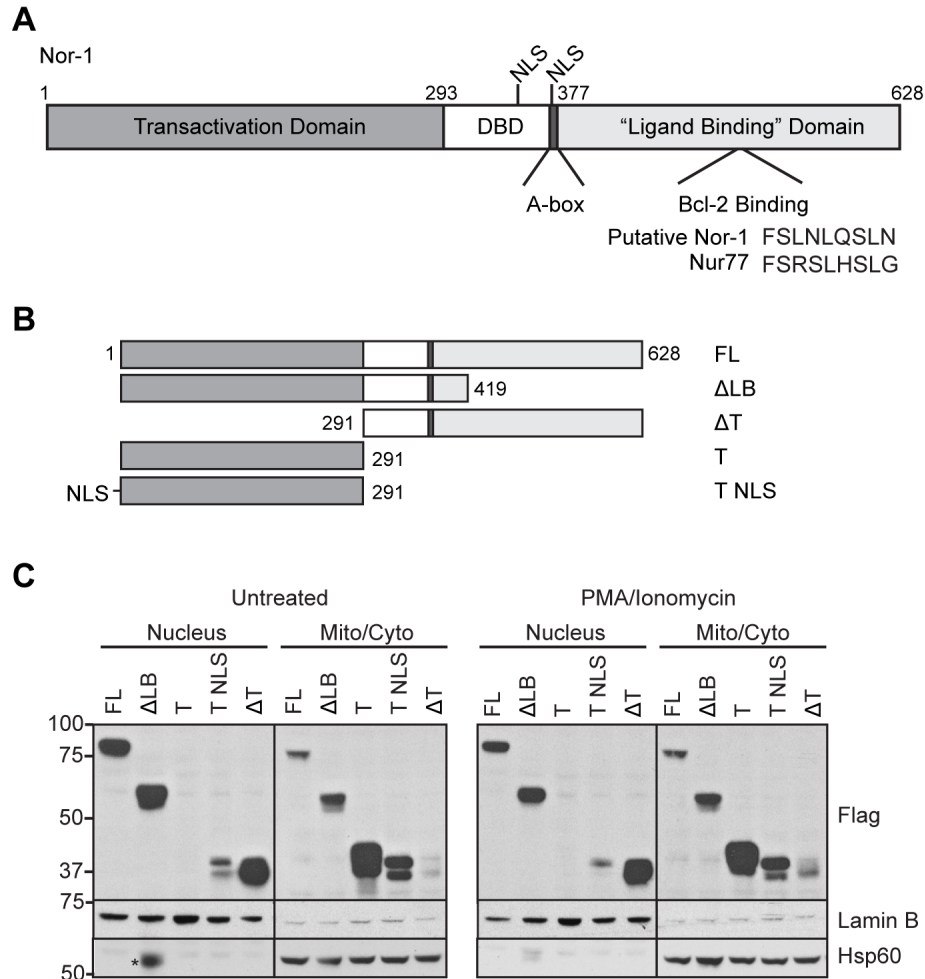


Figure 1. Nor-1 transactivation domain is necessary for localization outside the nucleus. (A) Schematic of the domain structure of mouse Nor-1. Two nuclear localization signals (NLSs) and an A-box, essential for DNA binding, are located within the central DNA binding domain. Flanking this domain are an N-terminal transactivation domain and a C-terminal "ligand binding" domain. Nor-1's putative Bcl-2 binding domain is located within the "ligand binding" domain. (B) Diagram of Nor-1 truncation mutants with cuts made at the boundaries between Nor-1's protein domains. Transactivation domain-only mutants (T and T NLS) were made with and without the addition of exogenous NLSs due to loss of the endogenous NLSs with deletion of the DNA binding domain. (C) Analysis of the nuclear versus mitochondrial/cytoplasmic localization of Flag-tagged Nor-1 mutants in 16610D9 cells by western blot. Cells were left untreated or TCR-stimulated for 24 hours with 60ng/ml PMA and 3μM Ionomycin. Fractionation purity was confirmed by blotting for Lamin B (nuclear) and Hsp60 (mitochondrial/cytoplasmic). Nor-1 ΔT localization was restricted to the nucleus, while Nor-1 T NLS was found mostly in the mitochondria/cytoplasm. Asterisks indicate residual bands.

Figure 2

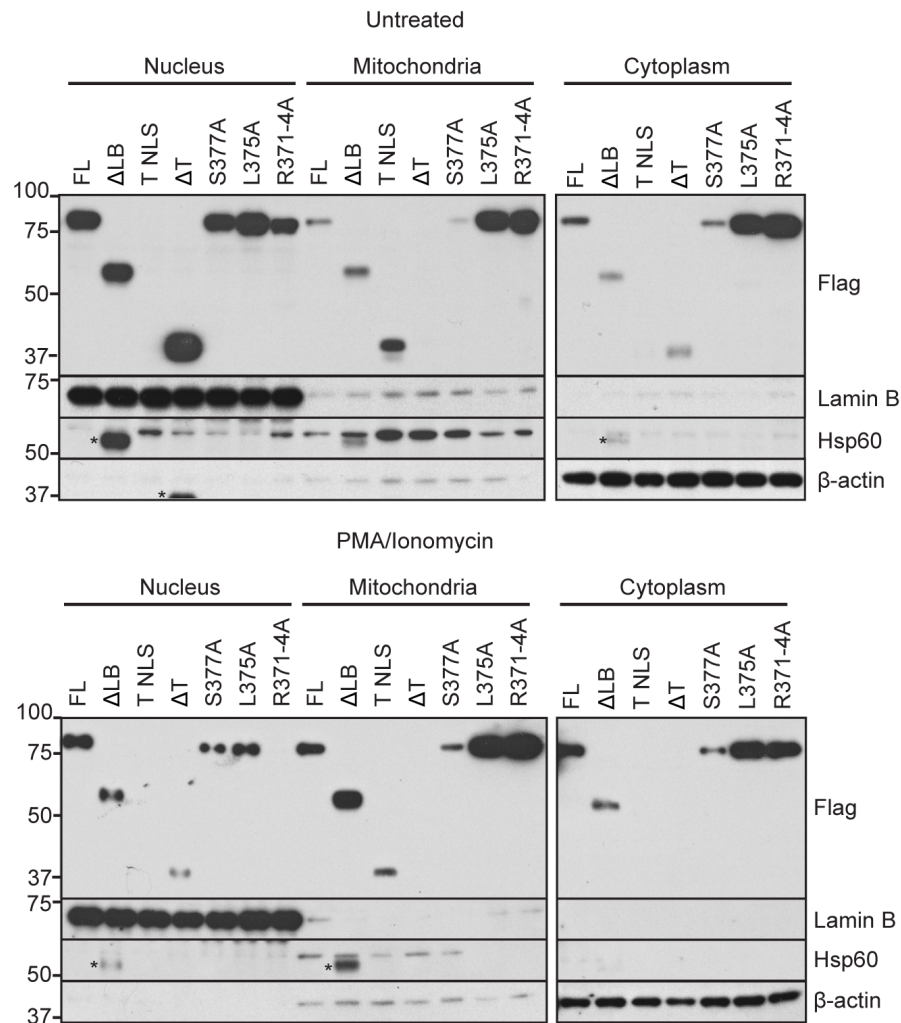
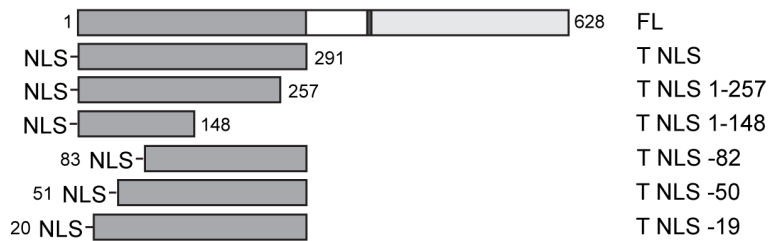


Figure 2. Nor-1 transactivation domain is necessary for localization to the mitochondria and regulation of Nor-1 DNA binding activity may control nuclear export. Analysis of the subcellular localization of Nor-1 mutants by an optimized fractionation procedure yielding a pure mitochondrial fraction. The localization of the Nor-1 truncation mutants in Figure 1 was reassessed along with three mutants containing point mutations within the DNA binding domain. Cells were left untreated or TCR-stimulated for 24 hours with 60ng/ml PMA and 3μM Ionomycin prior to fractionation. Nor-1 mutants were detected by Flag immunoblot. Fractionation purity was confirmed by blotting for Lamin B (nuclear), Hsp60 (mitochondrial) and β-actin (cytoplasmic). ΔT was again found primarily in the nucleus, while T NLS was only localized to the mitochondria, indicating that the transactivation domain is important for mitochondrial localization. Mutation of serine 377 to alanine (S377A) within the A-box does not effect translocation. Mutation of residues disrupting DNA binding (L375A) or DNA binding plus one NLS (R371-4A) promotes greater mitochondrial localization.

Figure 3

A



B

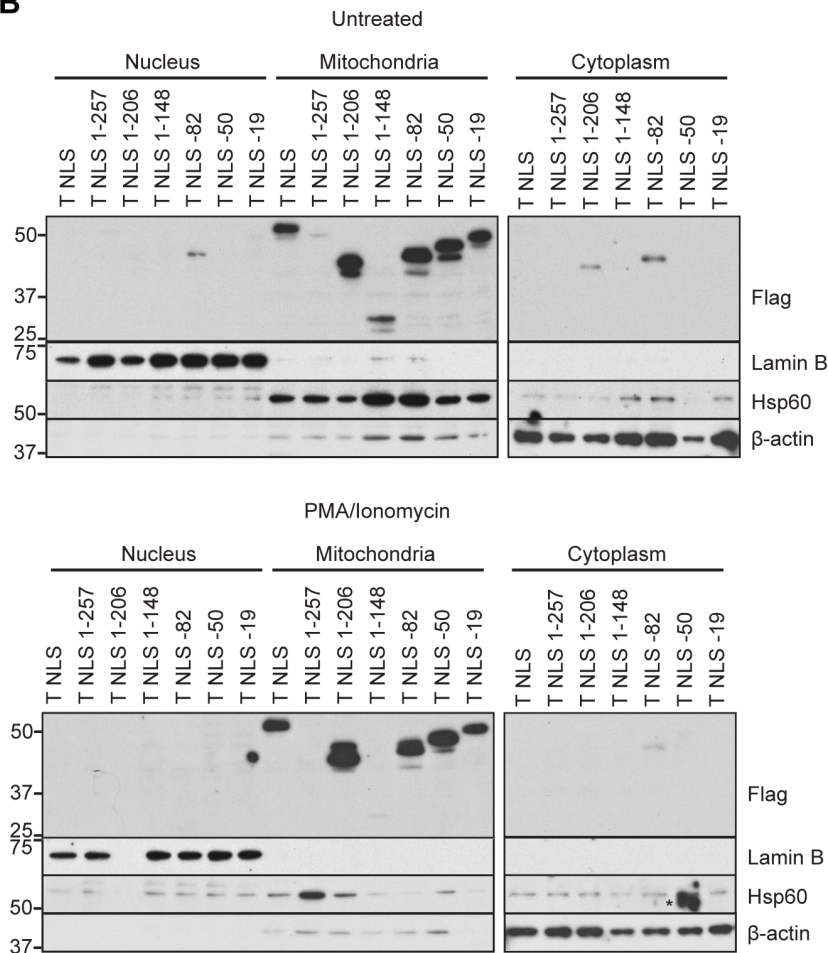
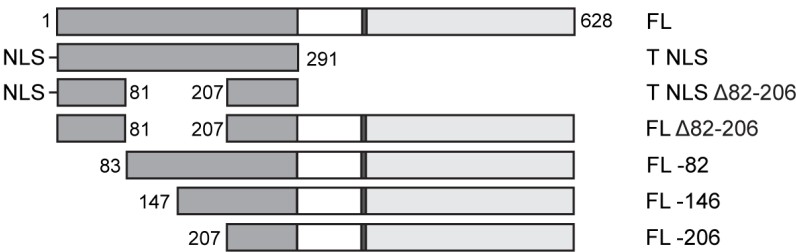


Figure 3. Nor-1 N-terminal amino acids 83-148 are sufficient for localization to the mitochondria. (A) Diagram of Nor-1 mutants made by serial truncation of the T NLS mutant. (B) Analysis of the subcellular localization of the T NLS mutants depicted in A by western blot. Cells were treated as described in Figure 2. T NLS 1-257 was not expressed and T NLS 1-148 was expressed poorly. All expressed T NLS mutants were primarily localized to the mitochondria. This suggests that the residues they have in common, 83-148, are involved in targeting Nor-1 to the mitochondria.

Figure 4

A



B

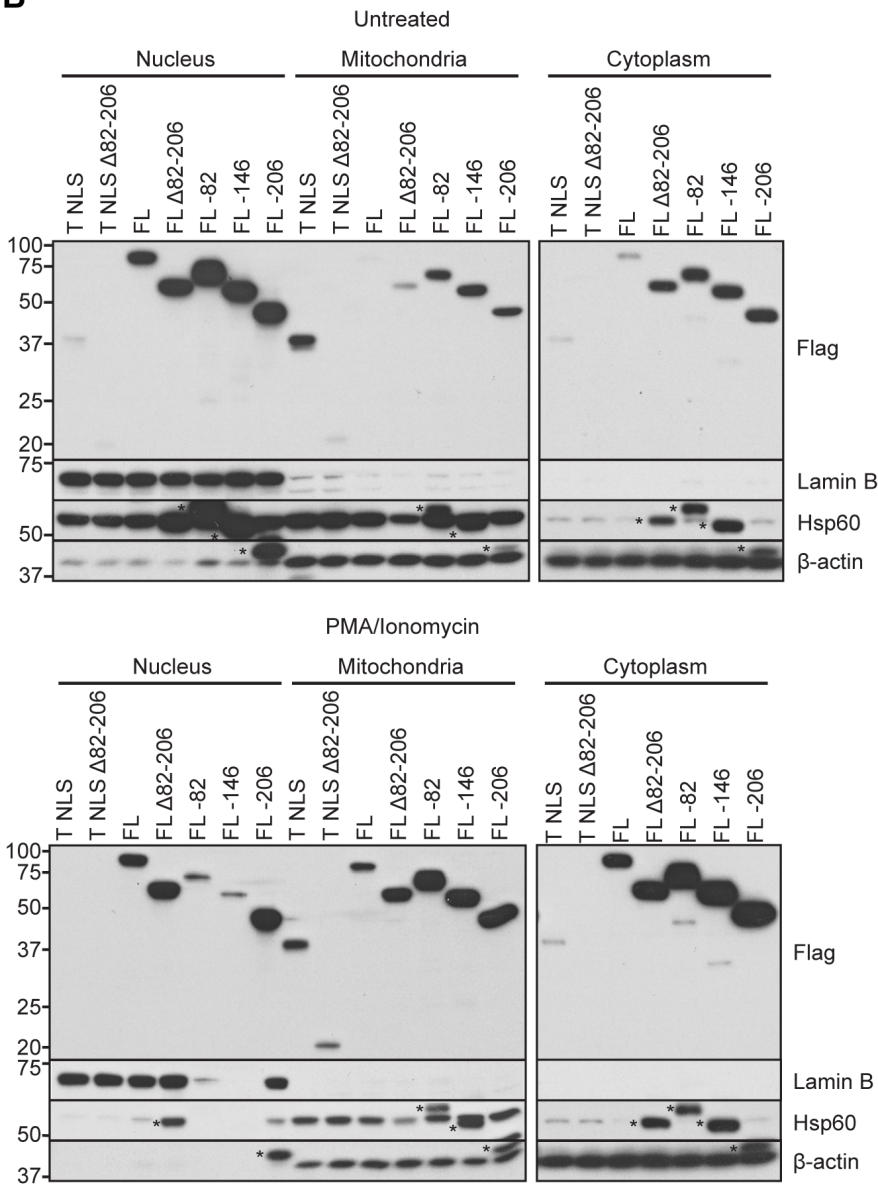
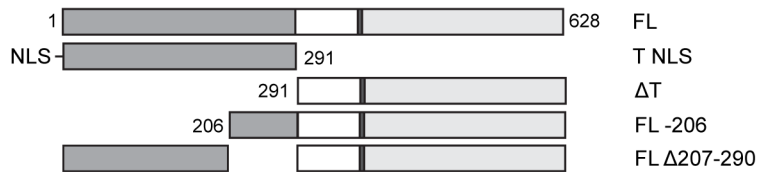


Figure 4. Residues 1-206 are not necessary for Nor-1 mitochondrial translocation. (A) Diagram of Nor-1 mutants created to assess the contribution of amino acids 82-206 to mitochondrial translocation. (B) Analysis of the subcellular localization of the Nor-1 mutants depicted in A by western blot. Deletion of residues 1-206 did not negatively impact Nor-1 T NLS or FL mitochondrial translocation.

Figure 5

A



B

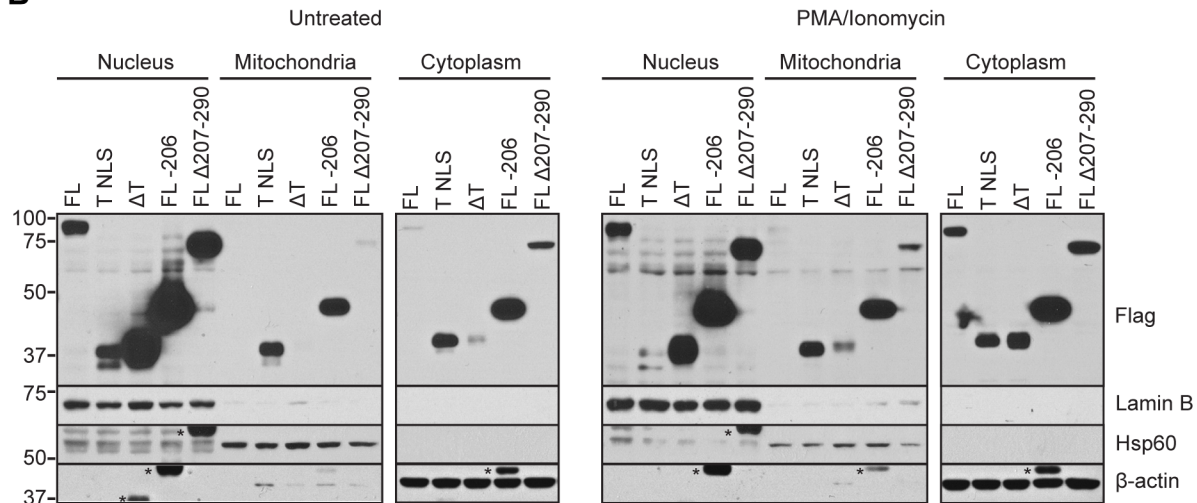


Figure 5. Residues 207-290 are sufficient, but not necessary for Nor-1 mitochondrial translocation. (A) Diagram of Nor-1 mutants used to assess the importance of residues 207-290 for Nor-1 mitochondrial translocation. (B) Analysis of the subcellular localization of the mutants depicted in A by western blot. FL -206 translocates better than FL, while ΔT is restricted to the nucleus, indicating that residues 207-290 are sufficient for translocation. Deletion of residues 1-206 also appears to relieve inhibition on translocation. FL Δ207-290 translocates the same as FL, indicating that residues 207-290 are not necessary for translocation.

Figure 6

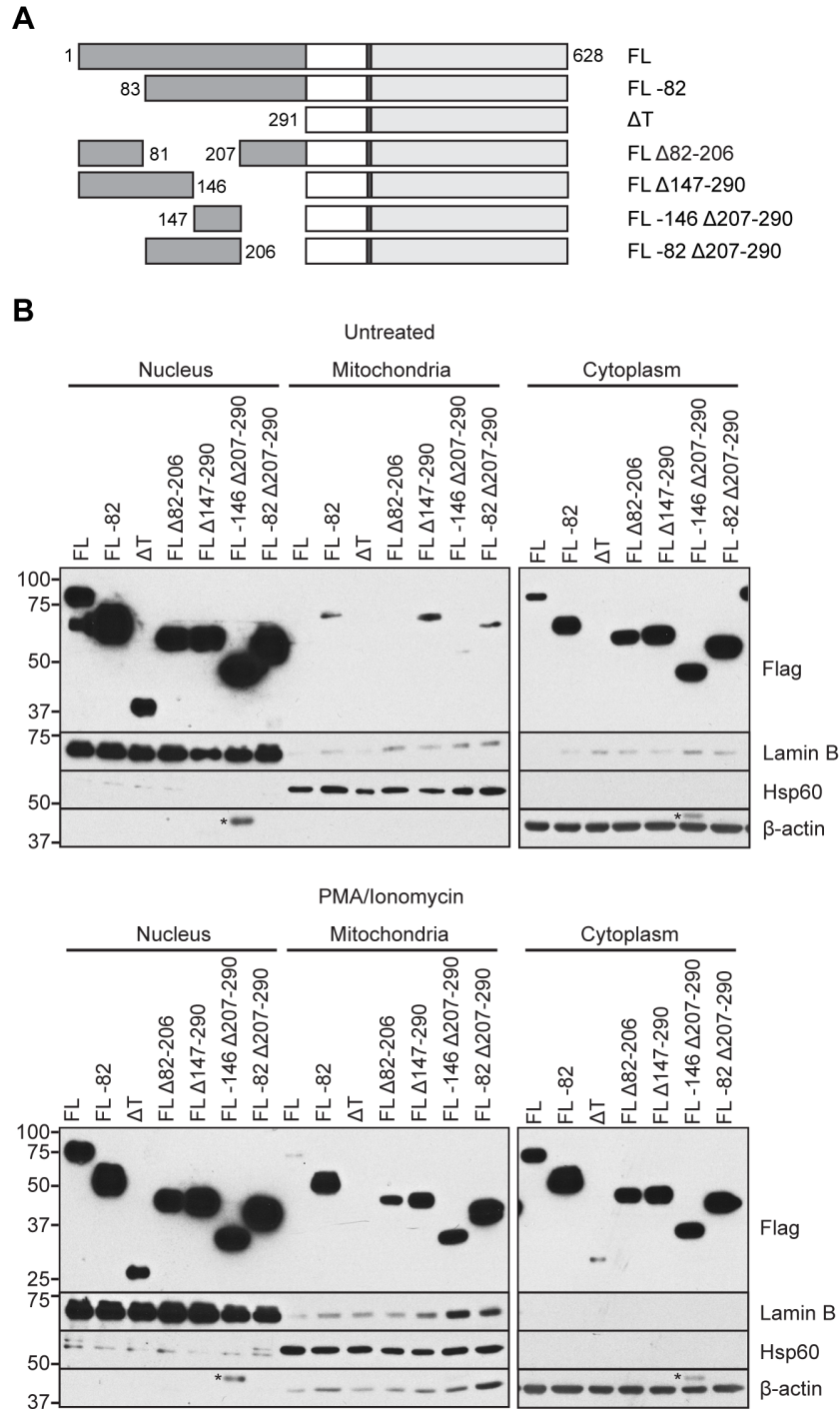


Figure 6. Regions 83-147, 148-206 and 207-290 are all independently sufficient for Nor-1 mitochondrial translocation. (A) Diagram of Nor-1 mutants created to identify smaller regions within 83-290 that are important for translocation. (B) Analysis of the subcellular localization of the mutants depicted in A. All new mutants are able to translocate, identifying three regions that are independently sufficient for translocation.

Figure 7

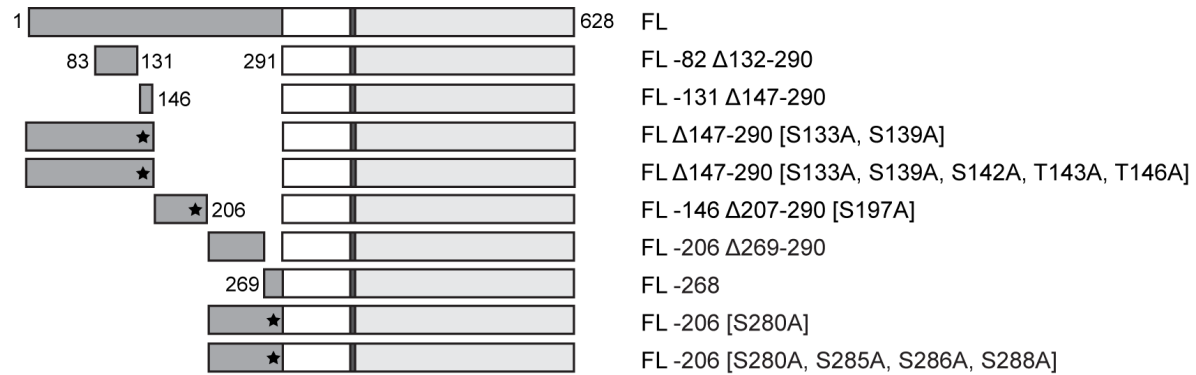
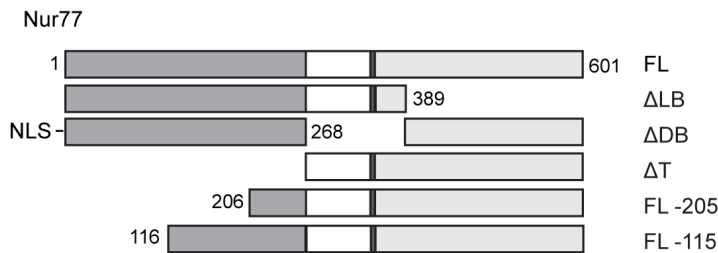


Figure 7. Nor-1 mutants to identify the residues within regions 83-147, 148-206 and 207-290 that are important for translocation. Diagram of the Nor-1 mutants created to narrow in on the residues critical for translocation. Stars indicate the general location of serine/threonine to alanine point mutations.

Figure 8

A



B

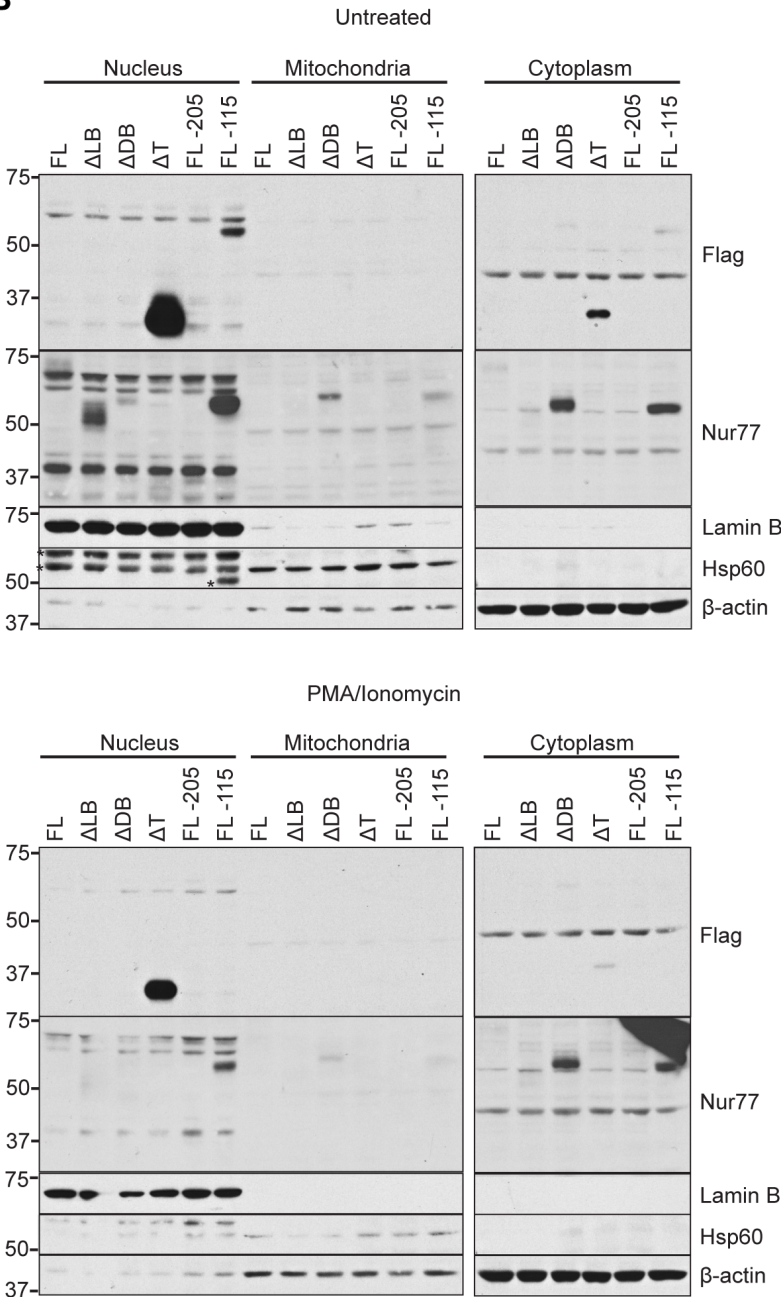


Figure 8. Similar to Nor-1, Nur77 transactivation domain is necessary for mitochondrial localization. (A) Diagram of Nur77 truncation mutants created to mirror some of the initial Nor-1 truncation mutants. Breakpoints were made at Nur77's protein domain junctions. (B) Analysis of the subcellular localization of the Nur77 mutants depicted in A by western blot. Nur77 mutants were detected by Flag and/or Nur77 immunoblot, due to issues detecting the Flag tag on some constructs. FL -205 was either not expressed or could not be detected by either antibody. Similar to Nor-1 ΔT , Nur77 ΔT localization was restricted to the nucleus. ΔLB was also restricted to the nucleus. ΔDB and -115 were able to translocate to the mitochondria. Asterisks indicate residual or non-specific bands.

Table 1

Construct	Forward Primer	Reverse Primer
FL	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
ΔLB	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCATCTG GAATAATCAAGATCTCTGGGTGT TGC
ΔT	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGACATGTGCCGTGTGCGGCCACAACGC	GACGAATTCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
T	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCAGCCT TCCCCAGATGATGAGCTCCTATT GG
T NLS	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGAAGAGACGCCGAAACCGAAAAGGGAG GAGAGGTCGTCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCAGCCT TCCCCAGATGATGAGCTCCTATT GG
L375A (Quikchange)	GGGAGGAGAGGTGCTGACCTTCCAAACCAAAGAGC CC	GGGCTCTTTGGTTTGGAAGGTG CACGACCTCTCCTCCC
S377A (Quikchange)	GGAGGAGAGGTGCTGCTGCCTGCCAAACCAAAGAGCC CACTAC	GTAGTGGGCTCTTTGGTTTGGC AGGCAGACGACCTCTCCTCC
R371-4A (Quikchange)	GACAGTCTGAAAGGGGCGCGGGTGCTCTGCCTTCC AAACCAAAG	CTTTGGTTTGGAAGGCAGAGCA CCCGCGGCCCTTTCAGACTGT C
T NLS -19	GACAGATCTGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGAAGAGACGCCGAAACCGAAAAGGGAG GAGAGGTCGTGACACTTATGGCTCGGAATACACCAC AG	GACGAATTCAGCCATCAGCCT TCCCCAGATGATGAGCTCCTATT GG
T NLS -50	GACAGATCTGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGAAGAGACGCCGAAACCGAAAAGGGAG GAGAGGTCGTGCCACTACATCCCTGCCCAGCTTC	GACGAATTCAGCCATCAGCCT TCCCCAGATGATGAGCTCCTATT GG
T NLS -82	GACAGATCTGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGAAGAGACGCCGAAACCGAAAAGGGAG GAGAGGTCGTGGGCCTCGGCCTTTGATCAAGATGG	GACGAATTCAGCCATCAGCCT TCCCCAGATGATGAGCTCCTATT GG
T NLS 1-257	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGAAGAGACGCCGAAACCGAAAAGGGAG GAGAGGTCGTCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCAGGAA GGTCAGCGTGCCGTCCTC
T NLS 1-206	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGAAGAGACGCCGAAACCGAAAAGGGAG GAGAGGTCGTCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCATGGA CTGGGCGCGGGAGGGTGTG
T NLS 1-148	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGAAGAGACGCCGAAACCGAAAAGGGAG GAGAGGTCGTCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCAGCCT GGAGTGGTCGGTGTAGAAGGC
FL -82	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGGGGCCTCGGCCTTTGATCAAGATGG	GACGAATTCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL -146	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCAGGCTTCCCCCGCAGGC	GACGAATTCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL -206	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGGCCGGCGGCCACCACTCGGCTA	GACGAATTCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
T NLS Δ82-206 Inside	ATGCCGCCTTCTGCCGGCGGCCAC	GTGGCCGCCGCAGAAAGGCGG CAT
T NLS Δ82-206 Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGAAGAGACGCCGAAACCGAAAAGGGAG GAGAGGTCGTCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCAGCCT TCCCCAGATGATGAGCTCCTATT GG
FL Δ82-206 Inside	ATGCCGCCTTCTGCCGGCGGCCAC	GTGGCCGCCGCAGAAAGGCGG CAT
FL Δ82-206 Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL Δ207-290 Inside	CCCAGTCCAGGCACATGTGCCGTG	CACGGCACATGTGCCTGGACTG GG

FL Δ207-290 Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL Δ147-290 Inside	ACACCGACCACTGGCACATGTGCC	GGCACATGTGGCAGTGGTCCGT GT
FL Δ147-290 Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL -146 Δ207- 290 Inside	CCCAGTCCAGGCACATGTGCCGTG	CACGGCACATGTGCCTGGACTG GG
FL -146 Δ207- 290 Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCAGGCTTCCCCCGCAGGC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL -82 Δ207- 290 Inside	CCCAGTCCAGGCACATGTGCCGTG	CACGGCACATGTGCCTGGACTG GG
FL -82 Δ207- 290 Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGGGGCTCGGCCTTTGATCAAGATGG	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL -82 Δ132- 290 Inside	CTGCCAGCGGCACATGTGCC	GGCACATGTGCCGCTGGGCAG
FL -82 Δ132- 290 Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGGGGCTCGGCCTTTGATCAAGATGG	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL -131 Δ147- 290 Inside	ACACCGACCACTGGCACATGTGCC	GGCACATGTGGCAGTGGTCCGT GT
FL -131 Δ147- 290 Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGACCTCCATGTACTTCAAGCAGTCTCCGC C	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL Δ147-290 [S133A, S139A] Inside	CAGCACCGCCATGTACTTCAAGCAGGCTCCGCCT	AGGCGGAGCCTGCTTGAAGTAC ATGGCGGTGCTG
FL Δ147-290 [S133A, S139A] Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL Δ147-290 [S142A, T143A, T146A] Inside	CCGCCTGCTGCACCGACCGCTCCAGG	CCTGGAGCGGTGGTGCAGCA GGCGG
FL Δ147-290 [S142A, T143A, T146A] Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL -146 Δ207- 290 [S197A] Inside	CTTCAAGCCCGCACCGCCAC	GTGGCGGTGCGGGCTTGAAG
FL -146 Δ207- 290 [S197A] Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCAGGCTTCCCCCGCAGGC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL Δ82-206 [S280A] Inside	CCAGCCTCCCAGCACCA	TGGGTGGTGTGGGAGGCTGG
FL Δ82-206 [S280A] Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL Δ82-206 [S285A, S286A, S288A] Inside	CCAATAGGGCCGCATCAGCTGGGGAA	TTCCCAGCTGATGCGGCCCTA TTGG
FL Δ82-206 [S285A, S286A, S288A] Outside	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGCCCTGCGTGCAAGCCCAGTATAGCC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC
FL -268	GACGAATTCGCCGCCGCCATGGACTACAAAGACGAT GACGACAAGTCCAGCCTGCTGGGAGAGAGCC	GACGAATTCCAGCCATCAGAAA GGCAGGGTGTCAAGGAAGAGC

Table 1. Primers used to generate Nor-1 mutant constructs. Inside and outside primers are indicated for site-directed mutagenesis procedures.

Chapter 4

Premalignant PTEN-deficient thymocytes activate microRNAs miR-146a and miR-146b as a cellular defense against malignant transformation

Summary

Cancer develops by a multi-step process during which cells acquire characteristics that allow them to evade apoptosis and proliferate unchecked. Sequential acquisition of genetic alterations drives this process, but also causes cellular stress, frequently prompting cells to enter a premalignant period during which they mount a defense against transformation. T cell-specific deletion of the tumor suppressor PTEN in mice induces premalignancy in the thymus and development of CD4⁺ T cell lymphomas in the periphery. Here we sought to identify factors mediating the cellular defense against transformation during the premalignant period. We identified several microRNAs upregulated specifically in premalignant thymocytes, including miR-146a, miR-146b and the miR-183/96/182 cluster. CD4-driven T cell-specific transgenic over-expression of *mir-146a* and *mir-146b* significantly delayed PTEN-deficient lymphomagenesis, and delayed c-myc oncogene induction, a key driver of transformation in PTEN-deficient T cell malignancies. We found that miR-146a and miR-146b targeting of Traf6 attenuates TCR signaling in the thymus and inhibits downstream NF- κ B-dependent induction of c-myc. Additionally, c-myc repression in mature CD4 T cells by miR-146b impaired TCR-mediated proliferation. Hence, we have identified two miRNAs that are upregulated as part of the cellular response against transformation, that when overrepresented can effectively inhibit progression to malignancy in the context of PTEN-deficiency.

Introduction

Malignant cancer is typically preceded by the development of premalignant lesions. However, not all premalignant lesions progress to malignancy⁹¹. Premalignant cells arise in large part due to genetic alterations that promote excessive growth and proliferation⁹². Characterizing changes in gene expression that occur in premalignant lesions could aid in correlating cellular responses to transformation with the risk of disease progression⁹². Furthermore, it could lead to identification of factors mediating the anti-tumor response that might inspire the design of more effective targeted therapeutics. While the biological heterogeneity of human premalignant lesions makes them difficult to study, premalignant lesions have been identified in many genetic mouse models of human cancer. The genetic homogeneity of mice can lead to more synchronous development of homogenous lesions, greatly facilitating the study of premalignancy⁹³.

Phosphatase and Tensin Homolog (PTEN) is one of the most frequently inactivated tumor suppressors in human cancer⁹⁴. PTEN dephosphorylates 3-phosphoinositide products of PI3 kinase (PI3K), thereby negatively regulating PI3K-Akt signaling, which promotes cell growth and proliferation^{95,96}. *Pten* is inactivated in many cancers by loss of heterozygosity, mutation or deletion, with high incidence in glioma, breast cancer, melanoma, prostate cancer, leukemia and lymphoma⁹⁴. In T cell acute lymphoblastic leukemia/lymphoma (T-ALL), *Pten* mutations have been identified in up to 27% of patients analyzed⁴⁷. Deletions are found in greater than 8% of patients and have been associated with early treatment failure^{47,97}. Consequential hyperactivation of the

PI3K-Akt pathway is also commonly observed⁹⁸. In cases where *Pten* is not altered at the genomic level, PTEN expression is still frequently downregulated⁹⁷.

T cell-specific deletion of PTEN in mice induces premalignancy in CD4⁺CD8⁺ (Double Positive, DP) thymocytes, which progresses to CD4⁺ T cell lymphoma in the lymph nodes and spleen^{51,99,100}. Interestingly, premalignancy occurs only in 6 weeks or older animals. In younger mice, T cell development in T-cell specific PTEN deficient mice (tPTEN^{-/-}) is completely normal with no signs of malignancy⁵¹. Premalignant thymocytes harbor T cell receptor (TCR) signaling-dependent *TCRa/c-myc* chromosomal translocations that promote c-myc upregulation, critical for promoting transformation of these cells⁹⁹. Premalignancy is also characterized by increased activation of the PI3K-Akt signaling pathway and induction of a senescence program⁵¹. However, since DP thymocytes do not proliferate to any significant degree, it is unlikely that senescence acts as a barrier to transformation in this model⁵¹. Thus, we sought to identify other factors that stave off transformation in premalignant PTEN-deficient DP cells.

Through microRNA (miRNA) expression analyses, we have identified miRNAs miR-146a and miR-146b as being significantly upregulated in premalignant DP cells of tPTEN^{-/-} mice. Strikingly, T cell-specific expression of *mir-146a* and *mir-146b* transgenes significantly delayed tPTEN^{-/-} lymphomagenesis, supporting their expression as a barrier to transformation. Tumor suppression was mediated by miR-146 attenuation of TCR signaling through repression of its target Traf6, an important activator of NF-κB. Our results not only support the potential therapeutic applications of these tumor suppressive miRNAs, but also suggest a general strategy for the identification of miRNAs that inhibit transformation in other cancer models.

Results

Upregulation of several miRNAs correlates with premalignancy in PTEN-deficient DP thymocytes

To identify miRNAs that might act to inhibit transformation of PTEN-deficient DP thymocytes, we performed a miRNA microarray on sorted DP cells from three 9-week-old tPTEN^{-/-} mice and three littermate controls (Figure 1A). 9-week-old tumor-free tPTEN^{-/-} mice were selected based on our prior correlation of premalignancy with this age⁵¹. We observed that 43 of 599 miRNAs were differentially expressed in the tPTEN^{-/-} samples. qRT-PCR validation of these results indicated that several miRNAs were upregulated specifically in 9-week tPTEN^{-/-} DP and sometimes CD4 SP thymocytes, but not in the corresponding mature T cells (Figure 1B, supplemental Figure 1A). Interestingly, upregulation was not detected in DP thymocytes from 5-week-old mice, where premalignancy has not yet occurred, indicating that these miRNAs are specifically induced during the premalignant period. We identified miR-146a as one of the most highly upregulated miRNAs in 9-week tPTEN^{-/-} DP thymocytes. Expression of highly homologous family member miR-146b was also increased. Though encoded on different chromosomes, miR-146a and miR-146b have identical seed sequences and differ by only 2 nucleotides at the 3' end, and therefore are predicted to have similar mRNA targets. Also highly upregulated in 9-week-old DP thymocytes were the miRNAs of the miR-183/96/182 cluster, miR-151-5p and miR-342-3p (supplemental Figure 1A).

We hypothesized that one or more of these miRNAs might be upregulated to inhibit malignant transformation of PTEN-deficient DP thymocytes.

Analysis of a larger cohort of 9-week-old tPTEN^{-/-} mice, however, revealed that these miRNAs are not always as highly upregulated at this age, consistent with the more modest phenotype observed for sample tPTEN^{-/-} 2 in the miRNA microarray. Analysis of c-myc expression in these mice uncovered a striking correlation between miRNA upregulation and high *c-myc* expression (Figure 1C)⁹⁹. *c-myc* upregulation was only observed in DP thymocytes and not in more mature thymocytes or T cells, paralleling the miRNA expression pattern we observed (Figure 1D). However, it is unlikely that c-myc drives expression of these miRNAs as prior analysis of c-myc regulation of global miRNA expression by others found that c-myc activation does not increase their expression^{101,102}.

In 12-week-old tPTEN^{-/-} mice harboring CD4⁺ T cell lymphomas, miR-146a and -146b expression in sorted CD4⁺ mature T cells was often downregulated (Figure 1E). This expression pattern is consistent with that observed for tumor suppressive miRNAs, such as miR-34a or let-7, as their downregulation is advantageous to the tumor¹⁰³. In contrast, the miR-183/96/182 cluster was further upregulated in tumor cells (data not shown).

Generation of T cell-specific miRNA transgenic mice

To test whether upregulation of miR-146a or miR-146b might act to inhibit malignant transformation of tPTEN^{-/-} DP thymocytes, we generated T cell-specific *mir-146a* and *mir-146b* transgenic mice. The miRNA transgenes were expressed under the control of the CD4 regulatory elements as previously described^{66,104}. The transgenic mice were generated on the C57BL/6 background and two *146a* founder lines (a1 and a8) and one *146b* founder line were used for analysis.

By RNase protection assay, we observed that miR-146a is normally expressed at low levels in thymocytes. In the *mir-146a* transgenic mice, miR-146a expression is increased to a level similar to that of miR-16, a miRNA that is abundant in thymocytes (Figure 2A)¹⁰⁵. This increase in miR-146a expression is lymphoid tissue-specific, as no upregulation was observed in the liver or kidneys (supplemental Figure 2A). The highest levels of *mir-146a* transgene expression were detected in DP thymocytes, followed by CD4 SP thymocytes and CD4⁺ mature T cells (Figure 2B). Some *mir-146a* transgene expression was also observed in CD4⁻CD8⁻ (Double Negative, DN) thymocytes and CD8⁺ mature T cells. Importantly, the level of miR-146 transgene expression in DP thymocytes was similar between all of the transgenic lines analyzed (Figure 2C).

***mir-146a* and *mir-146b* transgene expression inhibits tPTEN^{-/-} lymphomagenesis**

To determine whether miR-146a and miR-146b over-expression can inhibit PTEN-deficient lymphomagenesis, the *mir-146a* and *mir-146b* transgenic lines were crossed to the tPTEN^{-/-} mice. In preparation for the cross, the tPTEN^{-/-} mice were backcrossed to C57BL/6 to match the background of the miRNA transgenic mice. The change in background somewhat lengthened the lifespan of the tPTEN^{-/-} mice, with most mice succumbing to lymphoma by 20 weeks instead of 15 weeks. Strikingly, miR-146a or miR-146b over-expression significantly prolonged the survival of the tPTEN^{-/-} mice, with a median increase in survival of 8-10 weeks (Figures 2D and 2E).

To elucidate the mechanism by which miR-146a and miR-146b delay tumorigenesis, we first evaluated whether over-expression of the miRNAs alters T cell development. T cell development occurs normally in the *mir-146a* transgenic lines, with no significant differences in T cell proportions in the thymus or lymph nodes on wild-type (WT) or tPTEN^{-/-} backgrounds (supplemental Figure 2B). *mir-146b* transgenic mice had normal thymic cell proportions, however CD4 T cell proportions were consistently reduced, with a 2-fold decrease in CD4 T cell number in the lymph nodes regardless of PTEN status (Figure 2F). The *mir-146b* transgenic CD4 T cells had a modest but consistent reduction in surface expression of CD62L, which is normally upregulated upon thymic egress to mediate homing to the lymph nodes and spleen (Figure 2G). Reduced T cell homing to the lymph nodes might contribute to the lower *146b* CD4 T cell numbers. In contrast, *146a8* CD4 T cells expressed normal levels of CD62L (supplemental Figure 2C). *146b* CD4 T cell maturation was otherwise generally unaffected, with appropriate upregulation of Qa-2 and downregulation of HSA. CD62L levels in SP thymocytes were also largely normal (supplemental Figures 2B and 2C). Thus, T cell development appears largely unaltered by *mir-146a* and *mir-146b* transgene expression, with the exception of a moderate reduction in CD4 T cells numbers in the *146b* transgenic mice.

miR-146 over-expression delays c-myc upregulation associated with premalignancy

To evaluate whether miR-146 over-expression delays tPTEN^{-/-} tumor formation, we first sought to characterize the premalignant state of tPTEN^{-/-} mice on the C57BL/6 background. Consistent with the increased survival of C57BL/6 tPTEN^{-/-} mice, the appearance of premalignant markers is delayed compared to tPTEN^{-/-} mice on a mixed background⁵¹. Beginning around 11 weeks, blasting, characterized by increased forward and side scatters, is apparent in the thymus, but not the lymph nodes of tPTEN^{-/-} mice (Early Premalignant, Figure 3A). A slight increase in phosphorylated-Akt (P-Akt) is also evident in CD4 SP thymocytes (Figure 3B). Progression from early to late premalignancy is signified by more pronounced blasting and high levels of P-Akt in DP and CD4 SP thymocytes, but not mature T cells (Figure 3B). Paralleling P-Akt levels, Ki67 expression is also increased in DP and CD4 SP thymocytes during late premalignancy (Figure 3B). Importantly, DP thymocytes normally express high levels of Ki67, even though they don't proliferate^{51,104}. Increased c-myc expression corresponds with late premalignancy, as increased *c-myc* mRNA levels are found only in thymocytes expressing high levels of both phosphorylated Akt and Ki67 (Figure 3C). Consistent with this observation, increased c-myc protein levels were observed in the thymus, but not in the lymph node T cells of 12-week-old late premalignant mice (Figure 4A). However, in 13.5-week-old tPTEN^{-/-} mice that have developed tumors, c-myc upregulation was apparent in both compartments. Interestingly, no c-myc upregulation was observed in two 13.5-week-old littermate *146a8*/tPTEN^{-/-} mice in either compartment (Figure 4A). We also did not observe *c-myc* upregulation at the transcript level in thymocytes of 14-15 week tumor-free *146a1*/tPTEN^{-/-} and *146b*/tPTEN^{-/-} mice compared to littermate tPTEN^{-/-} controls that had acquired tumors by this age (Figure 4B). These data suggest that miR-146 over-expression might act to delay c-myc upregulation associated with premalignancy. To more comprehensively assess the impact of miR-146 over-

expression on premalignancy, we assessed blasting, phosphorylated-Akt levels, Ki67 expression and thymic *c-myc* mRNA expression in a large cohort of 13.5 to 15-week-old littermate or age-matched tPTEN^{-/-} and 146/tPTEN^{-/-} mice. These mice were then classified as early premalignant, late premalignant or malignant based on the criteria outlined above. A large majority of the 146/tPTEN^{-/-} mice were found to be in early premalignancy, compared to tPTEN^{-/-} mice, which were primarily in late premalignancy or had already developed tumors (Figure 4C). These data suggest that miR-146 over-expression acts to prolong the survival of tPTEN^{-/-} mice by delaying progression to malignancy. Consistent with increased *c-myc* mRNA levels in the thymus of late premalignant/malignant 146/tPTEN^{-/-} mice, upregulation of *c-myc* expression and *TCRa/c-myc* translocations were found in 146/tPTEN^{-/-} tumor cells (Figures 4D and 4E). Thus, miR-146 over-expression can delay, but not abolish the hallmark events of tPTEN^{-/-} lymphomagenesis.

miR-146 negatively regulates TCR signaling in thymocytes

TCRa/c-myc translocations in PTEN-deficient thymocytes were previously demonstrated to be TCR signaling-dependent⁹⁹. In mature T cells, miR-146a expression is induced by TCR stimulation and miR-146a then acts in a negative feedback loop to repress TCR signaling^{106,107}. We hypothesized that miR-146a and miR-146b might similarly be induced by TCR signaling in DP thymocytes and act in a negative feedback loop to inhibit TCR signals necessary for *TCRa/c-myc* translocations/*c-myc* upregulation. Indeed, we found that miR-146a and miR-146b were significantly upregulated in TCR-stimulated DP thymocytes from both WT and tPTEN^{-/-} 5-week-old mice (Figure 5A, sno-234 normalized in supplemental Figure 3). However, miR-146b was upregulated less than miR-146a, consistent with less induction of miR-146b compared to miR-146a in premalignant tPTEN^{-/-} DP thymocytes. Since strong amplification of Akt signaling is observed in late premalignant thymocytes, we tested whether Akt signaling downstream of the TCR might be responsible for miR-146a and miR-146b upregulation. Inhibition of Akt signaling with PI3K inhibitor LY294002 almost completely abrogated miR-146a and miR-146b induction in TCR-stimulated DP thymocytes (Figure 5A, supplemental Figure 3). Thus, Akt signaling downstream of the TCR is likely responsible for miR-146a and miR-146b upregulation during premalignancy in tPTEN^{-/-} mice.

In retrovirally transduced mature T cells, miR-146a has been shown to attenuate TCR signaling by targeting Traf6, a ubiquitin ligase that plays a key role in activating NF-κB^{106,108}. We hypothesized that miR-146 might act similarly in thymocytes to dampen the TCR signals that drive *TCRa/c-myc* translocations. Indeed, we found that Traf6 expression is reduced in tPTEN^{-/-} thymocytes from premalignant and tumor-bearing mice (Figure 5B). Additionally, in both *mir-146a* and *mir-146b* transgenic mice, we observed a significant reduction in Traf6 expression at both the transcript and protein levels on WT and tPTEN^{-/-} backgrounds (Figure 5C, supplemental Figure 4). The reduction in Traf6 expression was not due to global downregulation of miR-146 target genes as the expression of Stat1, a reported target of miR-146a in T cells whose 3' UTR has only partial homology to the miR-146a seed sequence¹⁰⁹, was not downregulated in either thymocytes or mature T cells of the *mir-146a* transgenic mice (Figure 5D). We next tested whether Traf6 downregulation by miR-146 results in

decreased NF- κ B activation downstream of TCR signals by measuring degradation of the NF- κ B inhibitor I κ B α . Upon PMA and Ionomycin stimulation, we observed that I κ B α degradation was significantly attenuated in *mir-146* transgenic thymocytes (Figure 5E, supplemental Figure 4). At later time points, we also observed attenuated induction of the NF- κ B target gene c-myc (Figure 5E, supplemental Figure 4). Since *TCR α /c-myc* translocations in tPTEN^{-/-} premalignant thymocytes are dependent on intact TCR signaling, miR-146 attenuation of TCR signaling might reduce the incidence of these translocations, explaining the delay in c-myc upregulation we observe. Additionally, diminished endogenous c-myc levels due to NF- κ B inhibition might increase the threshold for survival and proliferation of transforming thymocytes.

Reduced proliferative capacity of *mir-146b* transgenic CD4 T cells

As shown above, T cell development in *mir-146a* and *mir-146b* transgenic mice is largely normal with exception of reduced CD4 T cells in the *mir-146b* transgenic mice. Since TCR signals are important for survival of mature T cells¹¹⁰, we wondered if miR-146b attenuation of TCR signaling might contribute to the lower T cell numbers. Strikingly, we found that *mir-146b* transgenic CD4 T cells were significantly impaired in their ability to expand in response to TCR stimulation (Figure 6A). This effect was amplified on the tPTEN^{-/-} background. Co-cultured CD8 T cell expansion was normal, likely due to the absence of transgenic *mir-146b* expression (Figure 6A). Surprisingly, *mir-146a* transgenic CD4 T cell expansion was completely normal (Figure 6B). This observation is in agreement with our finding that *mir-146a* transgenic mice have normal T cell proportions.

A defect in TCR-mediated T cell expansion could be caused by insufficient activation through the TCR. However, *mir-146b* transgenic CD4 T cells upregulated activation markers CD69 and CD25 normally (supplemental Figure 5). Alternatively, this defect could be caused by increased apoptosis. In response to TCR stimulation, we observed a modest increase in apoptosis of *146b* CD4 T cells (Figure 6C). However, we were unable to rescue the expansion defect with the pan-caspase inhibitor zVAD-FMK (supplemental Figure 6), suggesting increased cell death is not the critical defect in *146b* T cells. Interestingly, we found that *146b* CD4 T cells have a pronounced defect in proliferation, with a reduced ability to dilute CFSE over time (Figure 6D). Cell cycle analysis of BrdU-pulsed T cells revealed that this defect was due to inhibition of the G₀/G₁ to S-phase cell cycle transition (24hr and 48hr, Figure 7A). In mature T cells, NF- κ B activation is important for inducing expression of genes that drive the G₀/G₁ to S-phase transition, most importantly c-myc.¹¹¹ Interestingly, Traf6 expression was significantly decreased in *146b/tPTEN^{-/-}* and to some extent *146a/tPTEN^{-/-}* CD4 T cells (Figure 7B). Consistent with a reduction in NF- κ B activity, we found that c-myc levels were significantly reduced in *146b* CD4 T cells on both WT and tPTEN^{-/-} backgrounds (Figure 7C, supplemental Figure 7). Furthermore, increased expression of the cell cycle inhibitor p27 in naïve *146b* CD4 T cells and to lesser extent in *146a* CD4 T cells was observed (Figure 7C, supplemental Figure 7). Thus, decreased c-myc induction and high p27 levels likely cooperate to inhibit *146b* T cell proliferation.

Discussion

Inactivation of the tumor suppressor PTEN renders cells vulnerable to malignant transformation, however additional oncogene activation is frequently necessary to drive forward the transformation process⁹⁴. In human T-ALL, PTEN loss is frequently coupled with increased expression of the c-myc oncogene¹¹². Activating mutations of NOTCH1, which are found in 50% of T-ALL patients, also drive concurrent c-myc amplification and PTEN suppression^{97,113}. In mice, c-myc upregulation is observed in both T cell-specific and hematopoietic-specific PTEN-deficient mice^{99,114,115}, largely driven by the presence of *TCRa/c-myc* chromosomal translocations^{99,116}. In one model, concurrent deletion of c-myc completely abrogated leukemia/lymphoma development¹¹⁴. Thus, in both humans and mice, activation of c-myc plays a key role in promoting development of PTEN-deficient T cell malignancies. In our study, through analysis of premalignant lesions in tPTEN^{-/-} mice, we have identified two miRNAs that act to delay this crucial c-myc induction and inhibit development of lymphomas.

Through analysis of *mir-146a* and *mir-146b* T cell-specific transgenic mice, we found that both miRNAs act to suppress TCR signals in thymocytes. Intact TCR signaling is a prerequisite for *TCRa/c-myc* translocations in PTEN-deficient premalignant thymocytes⁹⁹. By dampening TCR signals, these miRNAs might act to decrease the incidence of translocations, thus delaying c-myc upregulation and thymocyte transformation. While much of what drives chromosomal translocations between particular genetic loci remains unclear, recent data suggests that translocations occur more frequently in genic regions that are expressed versus silent¹¹⁷. By targeting Traf6, miR-146 represses NF-κB-dependent transcription of c-myc downstream of the TCR and might thereby decrease the participation of the c-myc locus in translocations. Alternatively, reduced endogenous c-myc expression might increase the threshold for survival and proliferation of cells already containing genomic aberrations.

The perfect seed sequence homology between miR-146a and miR-146b predicts that these two miRNAs should have the same mRNA targets and function redundantly¹⁰³. Consistent with this, our studies indicate that both miRNAs act similarly to repress Traf6, thereby negatively regulating NF-κB signaling and c-myc induction. However, the magnitude of this phenotype was somewhat attenuated in *mir-146a* versus *mir-146b* transgenic mice. The impact of this was most apparent in mature T cells, where transgenic expression of *mir-146b*, but not *mir-146a*, resulted in decreased CD4 T cells numbers and impaired TCR-mediated proliferation. However, while this T cell phenotype has not been previously reported for miR-146b, retroviral over-expression of miR-146a in T cells was shown to impair TCR-mediated proliferation. Conversely, *mir-146a*-deficiency promotes hyperproliferation of T cells in response to antigen¹⁰⁶. Hence, there is likely a threshold of miR-146 activity necessary to disrupt normal T cell function. Nevertheless, miR-146a and miR-146b extend tPTEN^{-/-} survival to a similar extent, which implies that in the context of tumor formation and growth, the more modest degree of miR-146a activity is sufficient to mediate its anti-tumor effects. In further support of redundant function of these miRNAs, we also found that *mir-146a*-deficiency alone is not sufficient to accelerate lymphomagenesis in tPTEN^{-/-} mice (supplemental Figure 8).

Traf6 is a key activator of NF- κ B downstream of TCR signaling¹⁰⁸. Consistent with Traf6 being a primary target of miR-146 in thymocytes and T cells, miR-146 over-expression phenocopies aspects of NF- κ B deficiency in T cells, which results in decreased T cell numbers and/or impaired TCR-mediated proliferation and survival^{111,118-122}. This defect was attributed to inhibition of NF- κ B-dependent induction of c-myc, resulting in defective entry into S-phase¹¹¹. Similarly, in *mir-146b* transgenic CD4 T cells, we observed reduced c-myc induction and delayed S-phase entry in response to TCR stimulation. Thus, attenuation of NF- κ B signaling through targeting of Traf6 likely accounts for much of the *mir-146b* mature T cell phenotype. More modest targeting of Traf6 by miR-146a and differential regulation of p27 levels likely accounts for the normal phenotype of *mir-146a* transgenic T cells.

Our data support a tumor suppressive role for miR-146a and miR-146b in murine T cell malignancies mediated by repression of TCR signaling and NF- κ B activation. These observations are supported by recent analysis of *mir-146a*-deficient mice, which develop a multi-organ inflammatory disease starting at 6-8 months-of-age that culminates in the development of myeloid sarcomas and some B and T cell lymphomas^{123,124}. Consistent with miR-146 playing a tumor suppressive role in human myeloid malignancies, miR-146a was significantly downregulated in several studies profiling miRNA expression in acute and chronic myeloid leukemia (AML/CML) samples and myelodysplastic 5q⁻ syndrome¹²⁵⁻¹²⁹. Also, enforced expression of miR-146a in AML cells lines inhibited cell proliferation and increased sensitivity to anti-leukemic drugs¹²⁷. In support of a tumor suppressive role for miR-146 in human T cell malignancies, a comprehensive screen of miRNA expression in 50 primary T-ALL samples showed considerable downregulation of miR-146a expression in T-ALL samples compared to normal T cell controls¹³⁰. Interestingly, studies have shown that NF- κ B is often activated downstream of NOTCH1 in human T-ALL cell lines and treatment with the NF- κ B inhibitor bortezomib significantly inhibits their growth^{131,132}. Hence, miR-146 based therapeutics that act to down-modulate NF- κ B activity might be effective in treating T-ALL. In support of this notion and its potentially broader applicability, re-expression of miR-146a in cancer cell lines of non-hematopoietic tumor types in which NF- κ B is hyperactivated, such as pancreatic and breast cancer, has been shown to repress NF- κ B activity and reduce proliferative and invasive capacity^{133,134}.

Materials and Methods

Mice

Characterization of the *Lck-Cre Pten^{fl/fl}* (tPTEN^{-/-}) mice has previously been reported^{49,51}. Analyses were performed on a mixed 129Sv/J x CBA x C57BL/6 background or a C57BL/6 background (backcrossed more than 10 generations).

The miRNA transgenic mouse constructs were generated by cloning PCR-amplified pre-miRNA sequences with approximately 450bp of flanking sequence into the pTG4 construct^{66,104}. The primer sequences used were: miR-146a-forward ATCGATCGCTCGAGTCTGGCTAGCCTGG, miR-146a-reverse ATCGATCGCTCGAAATAGGCGTTGAG, miR-146b-forward ATCGATCGCTCGAGCCATAGGCTGTGAT, miR-146b-reverse ATCGATCGCTCGACCCACAGGAGGAT. Founders were identified by PCR genotyping

with miR-146a-forward, miR-146b-forward and miR-146a/b-reverse
TGTGCCAAAGGGATTTTAGG.

miRNA Microarray

DP thymocytes were collected by cell sorting. Total RNA was extracted with a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was submitted to Exiqon (Woburn, MA, USA) for quality control, labeling with miRCURY Hy3/Hy5 probes and hybridization onto a miRCURY LNA Array (v.11.0 *mmu*). Data analysis was performed by Exiqon (<http://mcb.berkeley.edu/labs/winoto/array.html>).

Quantitative Real-Time RT-PCR

DP and CD4 SP thymocytes were collected by cell sorting and mature T cells were collected by negative selection column purification or cell sorting. Taqman MicroRNA Assays (Applied Biosystems, Life Technologies, Grand Island, NY, USA) specific to each miRNA were used for miRNA quantitation. For mRNA quantitation, reverse transcription was performed using SuperScript III with Oligo(dT) primers (Invitrogen, Life Technologies). SYBR Green (Invitrogen) real-time PCR was performed using the following primers: GAPDH-forward
ATCTTCTTGTGCAGTGCCAGCCTCGTCCCG, GAPDH-reverse
AGTTGAGGTCAATGAAGGGGTCGTTGATGG, c-myc-forward
GCCCCTAGTGCTGCATGA, c-myc-reverse AGAAGATGAGGAAGAAATTGATGTGG,
Traf6-forward TTTCCCTGACGGTAAAGTGC, Traf6-reverse
CTGGCACTTCTGGAAAGGAC.

RNase Protection Assay

miR-146a and miR-16 probes were synthesized and ³²P-labeled using the *miRVana* miRNA Probe Construction Kit (Ambion, Life Technologies) with the miR-16 DNA oligonucleotides provided in the kit and the following miR-146a DNA oligonucleotides as templates: sense TGAGAACTGAATTCCATGGGTTCCTGTCTC, anti-sense GAAGAACTGAATTTACAGGTCCCTGTCTC. The *miRVana* miRNA Detection Kit was used for probe hybridization and RNase A/RNase T1 digestion. Protected fragments were run on a polyacrylamide gel and visualized by X-ray film. Yeast RNA was used as a negative control.

Fluorescence Activated Cell Sorting (FACS) and Flow Cytometric Analysis

Lymphoid organs were isolated and cells dissociated through 40µm cell strainers and red blood cell lysis was performed. Cells were stained with fluorochrome-conjugated antibodies (eBioscience, San Diego, CA, USA and BD Biosciences, San Jose, CA, USA) in 1% fetal bovine serum (FBS) in PBS and then transferred to 50% FBS RPMI media for cell sorting with the Cytopeia (Seattle, WA, USA) Influx Sorter or the DAKO-Cytomation (Carpinteria, CA, USA) MoFlo High Speed Sorter. For flow cytometric analysis, live cells were surface stained in 1% FBS in PBS with fluorochrome-conjugated antibodies (eBioscience and BD Biosciences). For Annexin V staining, after surface staining, cells were stained with FITC-conjugated Annexin V (BD Bioscience) in 1X Annexin V binding buffer (10X: 0.1M HEPES, pH 7.4; 1.4M NaCl; 25mM CaCl₂). For CFSE dilution analyses, T cells were labeled with 1µM Cell Trace CFSE (Invitrogen, Life

Technologies, Grand Island, NY, USA) according to the manufacturers protocol prior to stimulation. Cells were harvested and surface stained at the indicated time points. For intracellular staining, prior to surface staining, live/dead staining was performed in PBS using LIVE/DEAD Fixable Dead Cell Stain (Invitrogen). Cells were then fixed and permeabilized with the Cytfix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). Ki67 staining was performed with a PE-conjugated anti-Ki67 and isotype control antibody (BD Biosciences). For phosphorylated Akt staining, cells were incubated with 5% normal goat serum for 15 minutes prior to staining with anti-Ser473 phosphorylated Akt antibody (Cell Signaling, Danvers, MA, USA, clone 193H12 #4058) or a rabbit IgG isotype control antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour on ice. Cells were then stained with a PE conjugated goat anti-rabbit secondary antibody for 30 minutes (Imgenex, Novus Biologicals, Littleton, CO, USA). For BrdU/7AAD analysis, stimulated T cells were pulsed for one hour with 10 μ M BrdU prior to harvest. Cells were then live/dead stained, surface stained and fixed as described above. 1mg/ml DNase I (Sigma, St. Louis, MO, USA) in PBS was added to the cells for 1 hour at 37°C. Cells were then stained with FITC anti-BrdU (BD Biosciences) for 20 minutes at room temperature. Cells were resuspended in 10ul of 7AAD (BD Biosciences), followed directly by addition of 300ul of 1% FBS in PBS. All samples were analyzed on the BD Biosciences LSR Fortessa or LSR II.

***In Vitro* TCR Stimulation of Thymocytes and T Cells**

Lymphoid organs were isolated and dissociated through 40 μ m cell strainers into 10% FBS in RPMI media (L-glutamine, Sodium Pyruvate, Non-essential Amino Acids, Hepes, β -mercaptoethanol and Penicillin-Streptomycin, Corning, Manassas, VA, USA). T cells were purified by negative selection columns (T Cell Enrichment Columns, R&D). Purified T cells were stimulated with 1 μ g/ml plate-bound anti-CD3 and anti-CD28 (clones 2C11 and PV-1, UCSF Hybridoma Facility, San Francisco, CA, USA) or 100ng/ml PMA (Calbiochem, EMD Biosciences, Billerica, MA, USA) and 1 μ M Ionomycin (Sigma) for various times as indicated. Following stimulation, CD4 T cells were further purified by staining with PE-conjugated antibodies to CD8, CD19, CD11b, CD11c and Ter-119 (eBioscience and BD Biosciences) and removal of contaminating cells using an EasySep Immunomagnetic PE Positive Selection Kit (StemCell Technologies, Vancouver, BC, Canada). Total thymocytes or FACS sorted DP thymocytes were stimulated with 10 μ g/ml CD3 and 20 μ g/ml CD28 or 2.5ng/ml PMA and 0.5 μ M Ionomycin.

Western Blotting

Thymocytes and mature T cells were collected as described above. Cells were pelleted and washed with PBS before lysis in 1% NP-40 protein lysis buffer (150mM NaCl, 1mM EDTA, 50mM Tris-HCl, pH 7.6, 1mM NaVO₄, 1mM NaF, 1mM DTT, 1mM PMSF and Sigma protease inhibitor cocktail). Protein was quantified with the Bio-Rad (Hercules, CA, USA) DC Protein Assay. 20-50 μ g of total protein was loaded onto 10% SDS-PAGE gels. The protein was transferred to nitrocellulose membranes and blocked with 5% bovine serum albumin in 1% Tween Tris buffered saline. Membranes were probed with the following antibodies: c-myc, GAPDH (Cell Signaling), I κ B α (Cell

Signaling or Santa Cruz Biotechnology), p27, Stat1 (Santa Cruz Biotechnology), Traf6 (Millipore, EMD Biosciences, Billerica, MA, USA) and β -actin (Sigma).

Fluorescence In-Situ Hybridization

Lymph node cells were isolated from tumor mice and treated for 3 hours with 0.04 μ g/ml Colcemid (Gibco Karyomax, Invitrogen) in RPMI media to arrest the cells in mitosis. Cells were pelleted, washed with PBS and suspended in pre-warmed 0.075M KCl (37°C) added drop-wise over a slow vortex. Cells were incubated in a 37°C waterbath for 20min. Cells were fixed on ice with 3:1 MeOH:glacial acetic acid added 10 drops at a time, every 5 min, 3X. Volume was brought to 10ml by slowly adding the fixative to the side of the tube. KCl and MeOH:glacial acetic acid phases were mixed slowly with a Pasteur pipet. Cells were pelleted and washed 3 times with 500ul fixative and dropped onto polylysine-coated slides. Slides were allowed to dry overnight. Slides were then incubated in 2X saline sodium citrate (SSC) for 30min at 37°C, RNase A digested (100 μ g/ml in 2X SSC) for 1hr and pepsin digested (0.01% in 0.01N HCl) for 15min, both at 37°C in a humid chamber. Slides were then fixed with 4% formaldehyde in 2X SSC for 20min at room temperature. Custom FISH bacterial artificial chromosome (BAC) probes were obtained from Empire Genomics (Buffalo, NY, USA) and probe hybridization was performed according to their standard protocol. The following probes were used: 5-ROX labeled BACs RPCI-55P19 and RPCI-98D8 for c-myc and 5-Fluorescein labeled BACs RPCI-357G5 and RPCI-339J4 for TCR α . Three out of the four probes were reported previously¹¹⁵. DAPI was applied to the slides before coverslipping with Vectashield Mounting Media for Fluorescence (Vector Labs, Burlingame, CA, USA). Images were captured on a Nikon (Melville, NY, USA) Eclipse E800 microscope with a Cool Snap EZ camera (Photometrics, Tucson, AZ, USA) using Slidebook 5.5 software at 40X magnification.

Figure 1

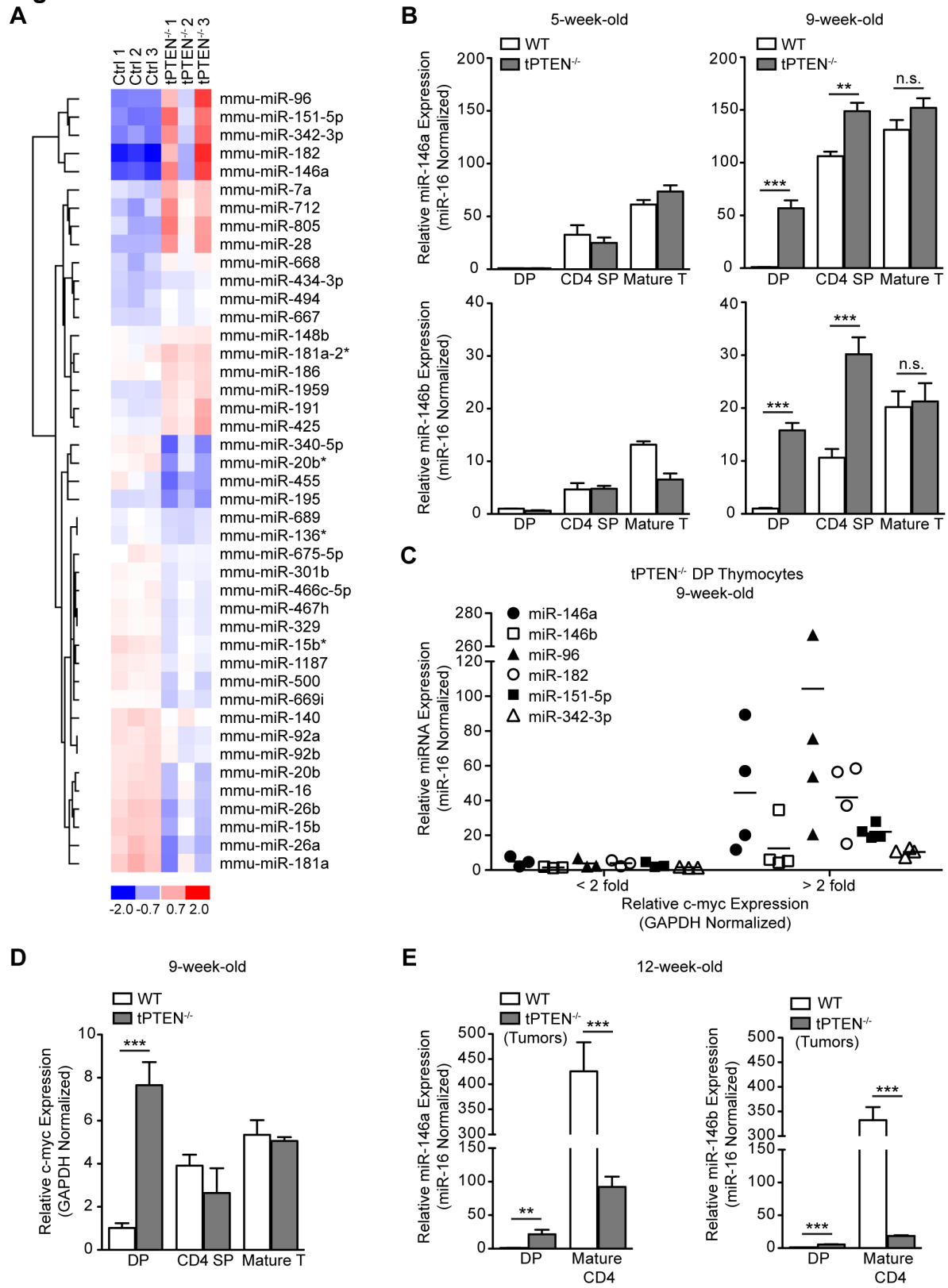


Figure 1. Several miRNAs are highly upregulated in premalignant tPTEN^{-/-} DP thymocytes. (A) Heat-map representing miRNAs with a combined p value of less than 0.05 and a delta log median ratio greater than 0.2. 43 out of 599 miRNAs evaluated were differentially expressed in tPTEN^{-/-} DP thymocytes from 3 premalignant 9-week-old mice compared to 3 littermate controls (129Sv/J x CBA x C57BL/6 background). Red and blue colors indicate miRNA expression levels above and below the mean, respectively. (B) qRT-PCR validation of the miRNA array results on FACS sorted DP and CD4 SP thymocytes and column purified mature T cells from 5-week-old and premalignant 9-week-old mice. miRNA expression levels are normalized to miR-16, which is expressed similarly in wild-type (WT) and tPTEN^{-/-} T cell subsets (supplemental Figure 1B). Fold change is relative to WT DP thymocytes. (C) qRT-PCR analysis of the correlation between miRNA and c-myc expression levels in 9-week-old tPTEN^{-/-} premalignant DP thymocytes. Fold change is relative to littermate WT DP thymocytes. c-myc expression is represented as less than or greater than a 2-fold increase over WT levels. (D) qRT-PCR analysis of c-myc expression in DP and CD4 SP thymocytes and mature T cells from premalignant 9-week-old mice with a pattern of miR-146a and miR-146b expression as shown in B. (E) miR-146a and miR-146b expression levels in thymocytes and lymph node tumor cells from 12-week-old mice (129Sv/J x CBA x C57BL/6 background). Fold change is relative to WT DP thymocytes. Data in B, D and E are presented as mean $\pm$ SD and are representative of 3 or more independent experiments. Statistics were calculated by Student's t-test: *** p < 0.001, ** p < 0.01, * p < 0.05, n.s. not significant.

Figure 2

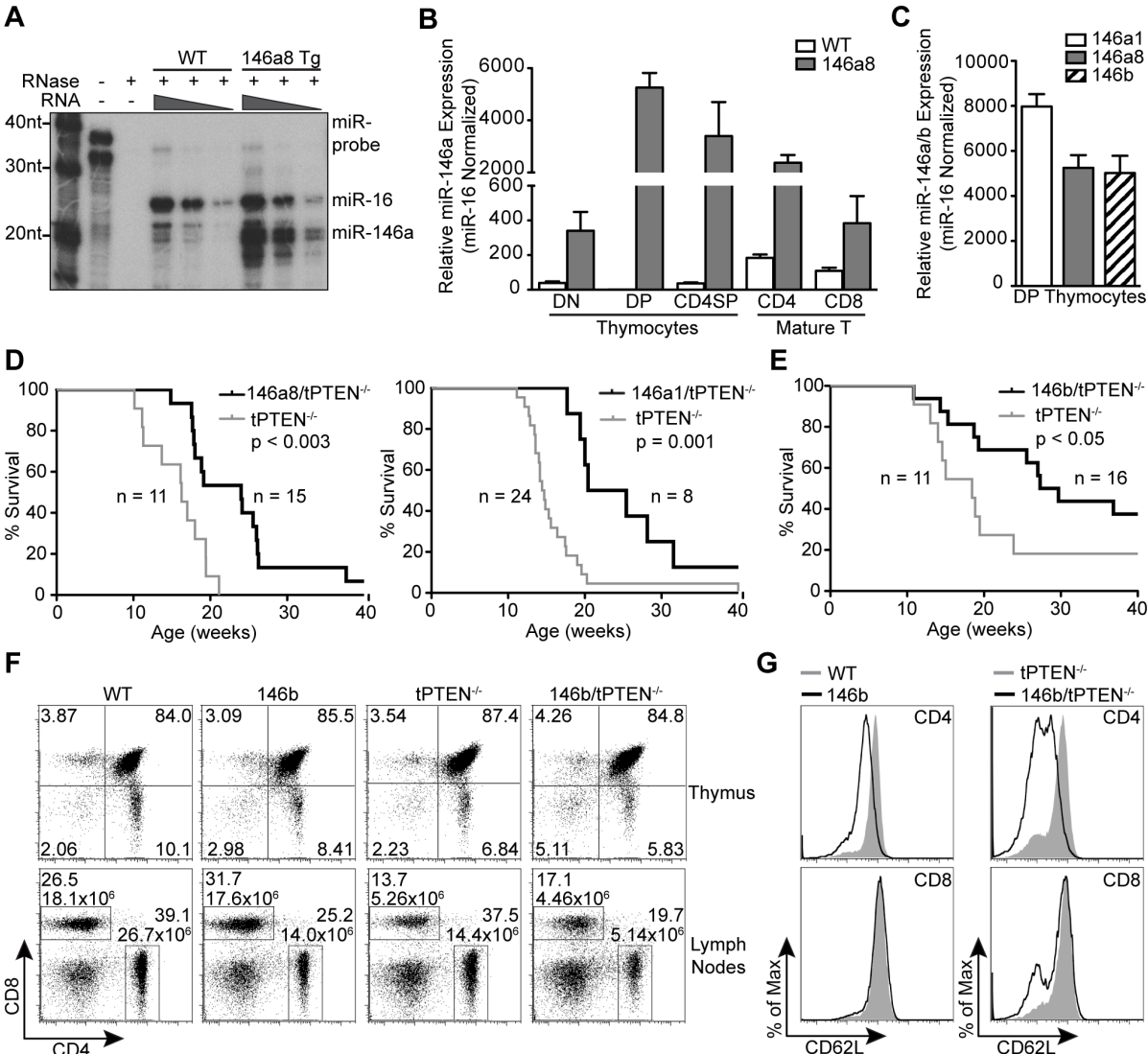


Figure 2. Transgenic expression of *mir-146a* and *mir-146b* delays tumorigenesis in tPTEN^{-/-} mice. (A) miR-146a expression levels in total thymocytes from *mir-146a* transgenic line 8 (*146a8*) versus wild-type (WT) mice by RNase protection assay. miR-16, a miRNA that is abundant in thymocytes, was used as a reference for miRNA levels. (B) qRT-PCR analysis of miR-146a expression levels in FACS sorted thymocyte and mature T cell subsets from *146a8* transgenic and littermate WT control mice. Fold change is relative to WT DP thymocytes and normalized to miR-16. (C) Comparison of miR-146 expression levels in DP thymocytes from all characterized *mir-146* transgenic founder lines. Fold change is relative to miR-146 expression in littermate WT controls. (D and E) Kaplan-meyer curves for the *mir-146a* transgenic lines *146a8* and *146a1* (D) and the *mir-146b* transgenic line (E) crossed to tPTEN^{-/-} mice on the C57BL/6 background. Statistical significance was calculated using the Gehan-Breslow-Wilcoxon method. (F) CD4 versus CD8 flow cytometric analysis of T cell populations in the thymus and lymph nodes of 7-week-old *146b* transgenic mice compared to littermate controls on both WT and tPTEN^{-/-} backgrounds. T cell numbers are indicated on the lymph node plots to reflect the decrease in CD4 T cell numbers with *mir-146b* transgene expression. Data are representative of 3 independent experiments. (G) Flow cytometric analysis of CD62L (L-selection) surface expression in lymph node T cells from 7-week-old *146b* transgenic and littermate control mice on WT and tPTEN^{-/-} backgrounds. Data are representative of 2 independent experiments.

Figure 3

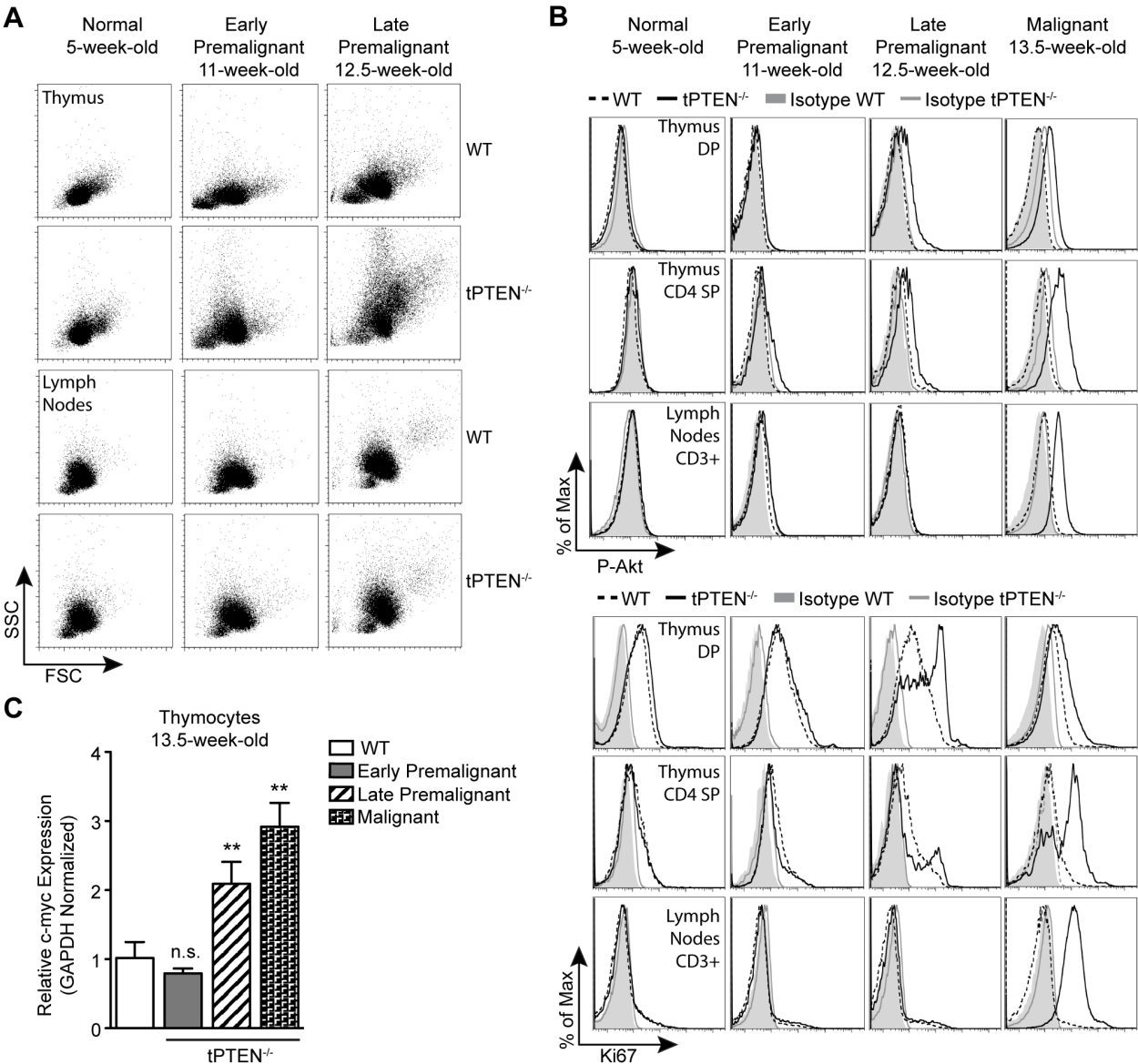


Figure 3. *c-myc* upregulation occurs in late premalignancy and coincides with increased Akt signaling and Ki67 expression. (A) Forward (FSC) and side scatter (SSC) analysis depicting increased blasting during premalignancy in the thymus, but not the lymph nodes of tPTEN^{-/-} mice compared to littermate WT controls. Blasting is more pronounced in late premalignancy. (B) Flow cytometric analysis of Akt phosphorylation at Ser473 (P-Akt) and Ki67 expression in DP and CD4 SP thymocytes and CD3+ lymph node T cells in littermate tPTEN^{-/-} and WT mice. Late premalignancy is defined by a marked increase in both phosphorylated Akt and Ki67 in DP and CD4 SP thymocytes, but low expression of both markers in lymph node T cells. (C) qRT-PCR analysis showing the correlation of *c-myc* upregulation with late premalignancy in total thymocytes from littermate 13.5-week-old tPTEN^{-/-} mice. Fold change is relative to WT thymocytes. Data are presented as mean $\pm$ SD and are representative of 3 or more independent experiments. Statistics were calculated by Student's t-test: ** $p < 0.01$, n.s. not significant.

Figure 4

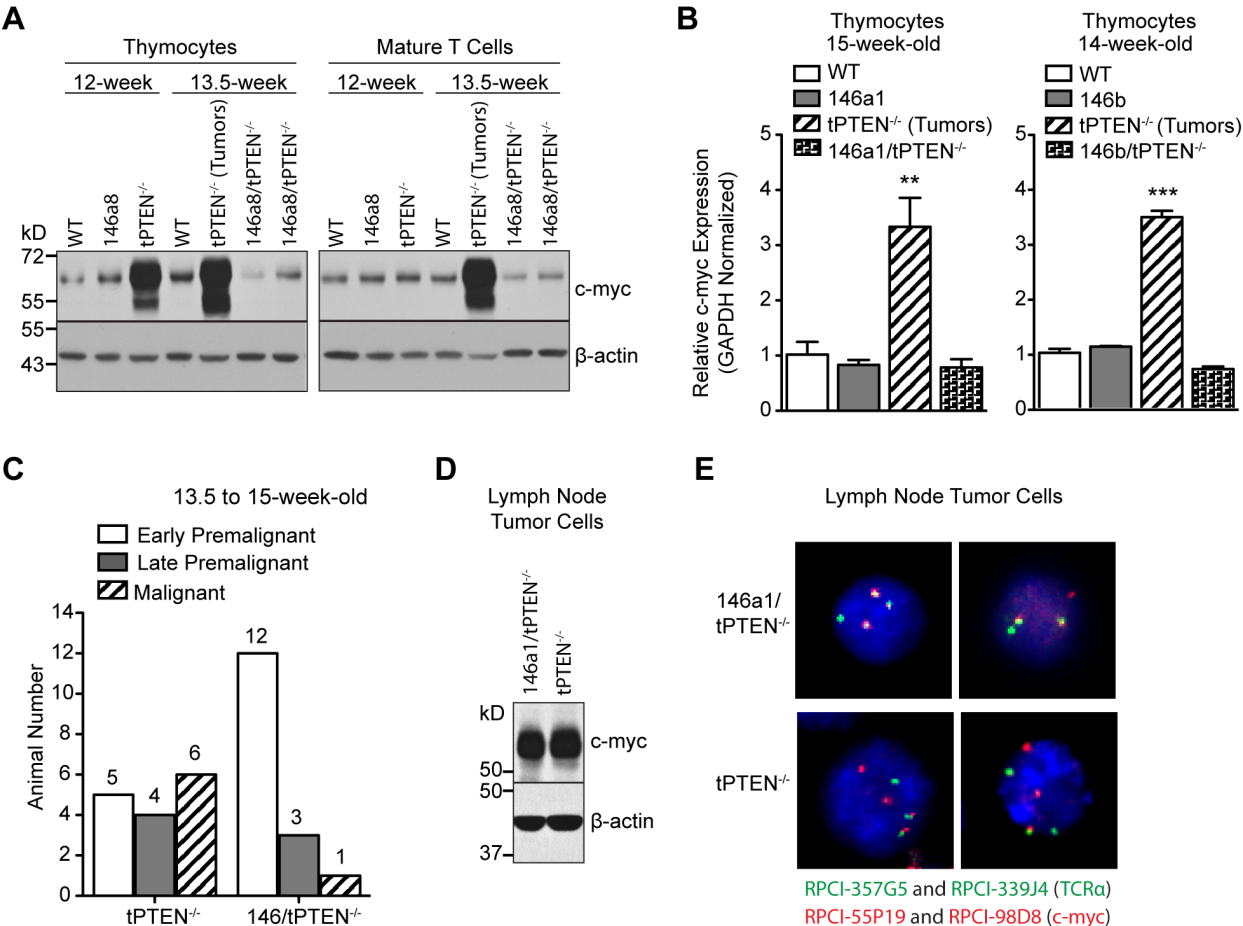


Figure 4. miR-146 over-expression delays c-myc upregulation associated with premalignancy. (A) Western blot analysis showing c-myc upregulation in total thymocytes from late premalignant 12-week-old tPTEN^{-/-} mice and both thymocytes and mature T cells from malignant 13.5-week-old tPTEN^{-/-} mice (C57BL/6 background). C-myc upregulation is not detected in the thymocytes or mature T cells of littermate 13.5-week-old 146a8/tPTEN^{-/-} mice. Data are representative of 2 independent experiments. (B) qRT-PCR analysis of *c-myc* expression in total thymocytes of 14 and 15-week-old mice. *c-myc* is upregulated in the thymocytes of tPTEN^{-/-} mice (with tumors), but not in thymocytes from tumor-free littermate 146a1/tPTEN^{-/-} or 146b/tPTEN^{-/-} mice. Data are presented as mean ± SD and are representative of 2 independent experiments per transgenic line. Statistics were calculated by Student's t-test: *** p < 0.001, ** p < 0.01. (C) Classification of littermate or age-matched 13.5 to 15-week-old tPTEN^{-/-} and 146/tPTEN^{-/-} mice based on the criteria outlined in Figure 3 (data from all transgenic lines were pooled). (D) Western blot analysis of c-myc protein expression in lymph node tumor cells of 12.5-week-old littermate tPTEN^{-/-} and 146a1/tPTEN^{-/-} mice. Data are representative of 3 independent experiments. (E) Fluorescence in-situ hybridization showing *TCRa/c-myc* chromosomal translocations in interphase-arrested lymph node tumor cells from the tPTEN^{-/-} and 146a1/tPTEN^{-/-} mice in D. Images were captured at 40X magnification.

Figure 5

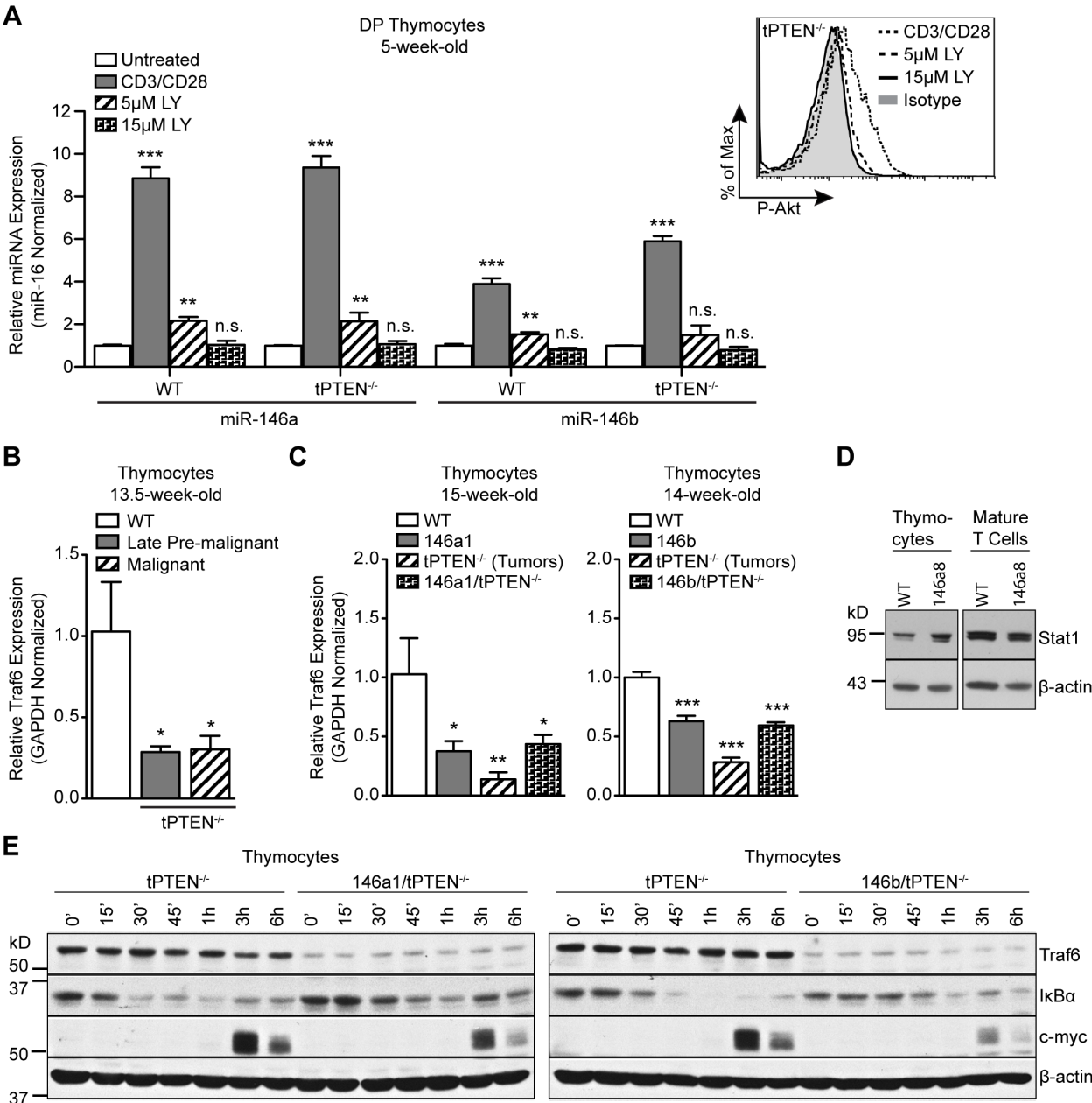


Figure 5. miR-146 represses TCR-dependent NF- κ B signaling in thymocytes by targeting Traf6. (A) qRT-PCR analysis of miR-146a and miR-146b expression levels in FACS sorted DP thymocytes, from 5-week-old littermate WT and tPTEN^{-/-} mice (C57BL/6 background), left unstimulated or stimulated with anti-CD3/CD28 antibodies for 16 hours. Where indicated, DP thymocytes were pre-treated with 5 μ M or 15 μ M LY294002 (LY) for 1 hour prior to anti-CD3/CD28 stimulation. Fold change is relative to untreated samples. Flow cytometry analysis shows dose-dependent inhibition of Akt phosphorylation in LY294002-treated cells. (B) qRT-PCR analysis of *Traf6* expression in littermate late premalignant and malignant tPTEN^{-/-} mice compared to an age-matched WT control (C57BL/6 background). (C) qRT-PCR analysis of *Traf6* expression in *146a1* and *146b* transgenic mice compared to littermate controls on WT and tPTEN^{-/-} backgrounds. (D) Western blot analysis of Stat1 protein expression in total thymocytes and mature T cells from 12-week-old littermate *146a8* transgenic and WT mice. (E) Western blot analysis of Traf6 expression, I κ B α degradation and c-myc induction in TCR-stimulated total thymocytes from 10-week-old *146a1*/tPTEN^{-/-} and *146b*/tPTEN^{-/-} mice and littermate or age-matched tPTEN^{-/-} controls. Thymocytes were stimulated with PMA and Ionomycin for the indicated times. Data in A-C are presented as mean $\pm$ SD and are representative of 3 independent experiments. Statistics were calculated by Student's t-test: *** p < 0.001, ** p < 0.01, * p < 0.05, n.s. not significant.

Figure 6

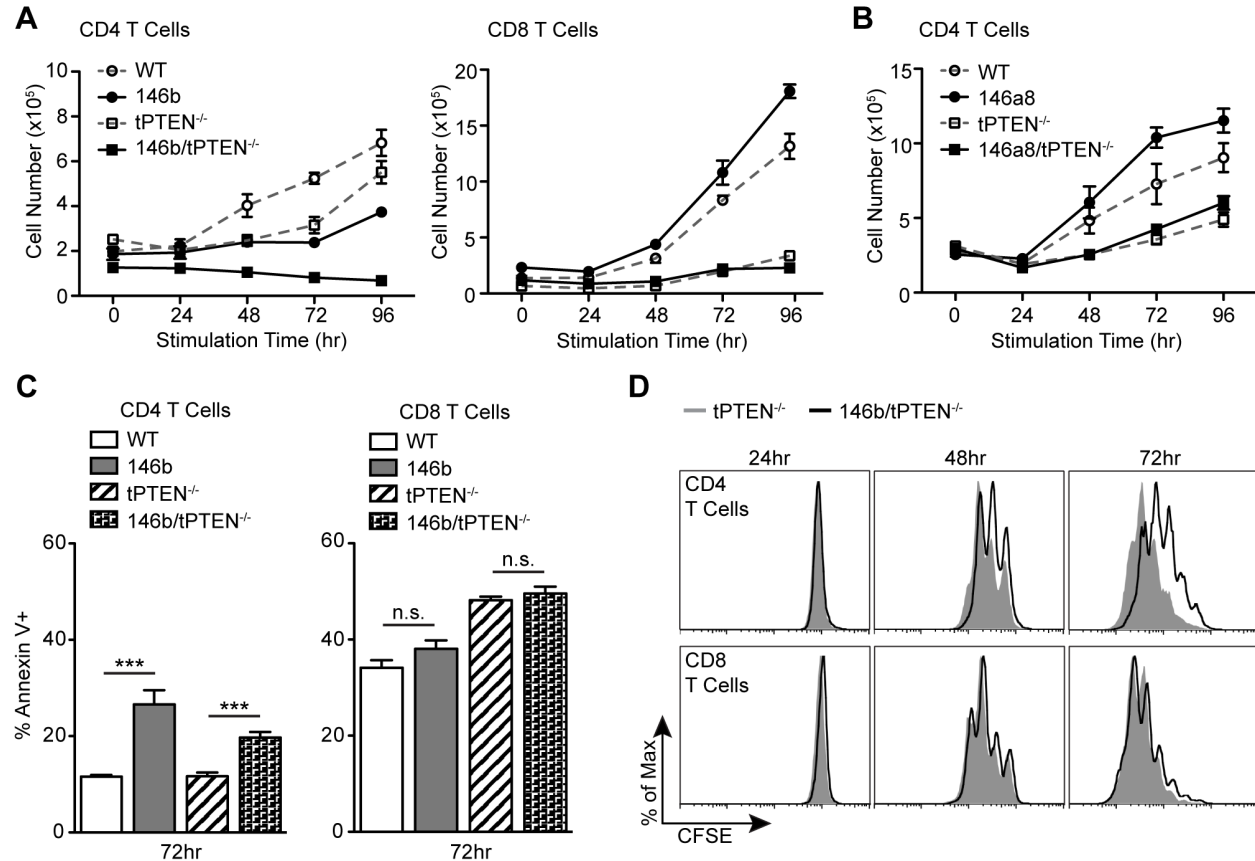


Figure 6. miR-146b negatively regulates CD4 T cell proliferation. (A) TCR-mediated expansion of CD4 and CD8 T cells from *146b* transgenic mice and littermate controls on WT and tPTEN^{-/-} backgrounds. Total T cells were stimulated with anti-CD3/CD28. Triplicate T cell samples were collected at each time point and total live T cell numbers were determined by Trypan blue exclusion. CD4 and CD8 numbers were calculated based on flow cytometric analysis at each time point. Data are representative of 4 independent experiments. (B) TCR-mediated expansion of CD4 T cells from *146a8* transgenic mice and littermate controls on WT and tPTEN^{-/-} backgrounds. The assay was performed as described in A. Data are representative of 2 independent experiments, one for each founder line. (C) Quantification of flow cytometric analysis of Annexin V+ T cells stimulated as in A for 72 hours. Data are representative of 2 independent experiments. (D) Flow cytometric analysis of CFSE dilution over time by T cells labeled with 1 μ M CFSE prior to stimulation as described in A. Histograms are representative of duplicate samples and 2 independent experiments. All mice used were littermate pairs between 6 and 10-weeks-old (C57BL/6). Data in A-C are presented as mean $\pm$ SD and statistics were calculated by Student's t-test: *** $p < 0.001$, n.s. not significant.

Figure 7

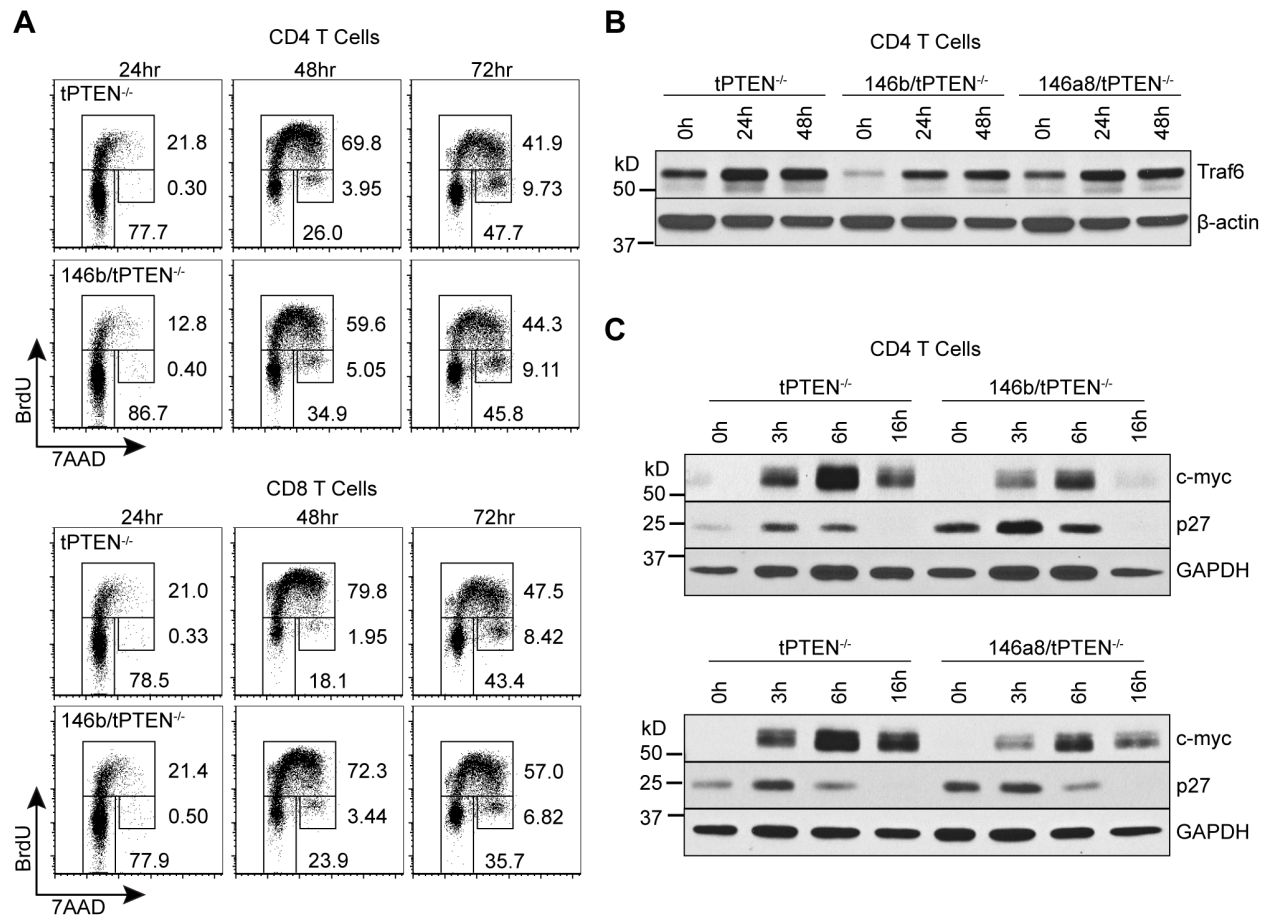


Figure 7. miR-146b negatively regulates the G₀/G₁ to S-phase cell cycle transition by targeting Traf6. (A) BrdU versus 7AAD flow cytometric analysis of T cells stimulated with anti-CD3/CD28 antibodies and pulsed with 10 μ M BrdU for 1 hour prior to harvest at the indicated time points. Plots are representative of duplicate samples. (B) Western blot analysis of Traf6 protein expression in purified CD4 T cells stimulated with anti-CD3/CD28 antibodies for the indicated times. (C) Western blot analysis of c-myc induction and p27 levels in purified CD4 T cells stimulated with anti-CD3/CD28 antibodies for the indicated times. Mice in A-C were littermate pairs between 6 and 8-weeks-old on the C57BL/6 background (146a8/tPTEN^{-/-} in B was age-matched). Data in A-C are representative of 2 independent experiments.

Figure S1

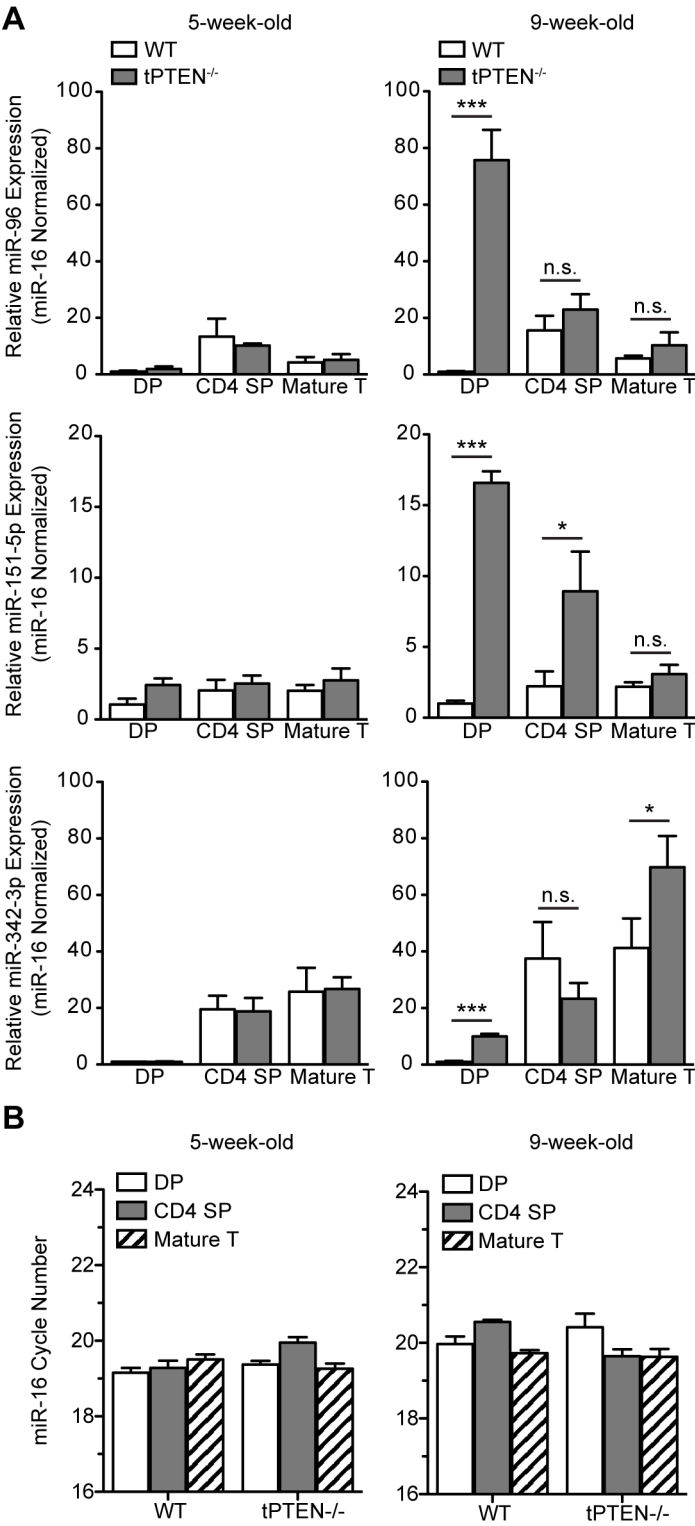


Figure S1. qRT-PCR validation of the miRNA microarray showing significant upregulation of several miRNAs in premalignant tPTEN^{-/-} DP thymocytes. (A) qRT-PCR analysis of miRNA expression in FACS sorted DP and CD4 SP thymocytes and column purified mature T cells from 5-week-old and premalignant 9-week-old mice (129Sv/J x CBA x C57BL/6 background). miRNA expression levels are normalized to miR-16. Fold change is relative to wild-type (WT) DP thymocytes. (B) qRT-PCR absolute cycle numbers for miR-16 demonstrating similarity in miR-16 expression between thymocyte and mature T cells subsets of 5 and 9-week-old WT and tPTEN^{-/-} mice.

Figure S2

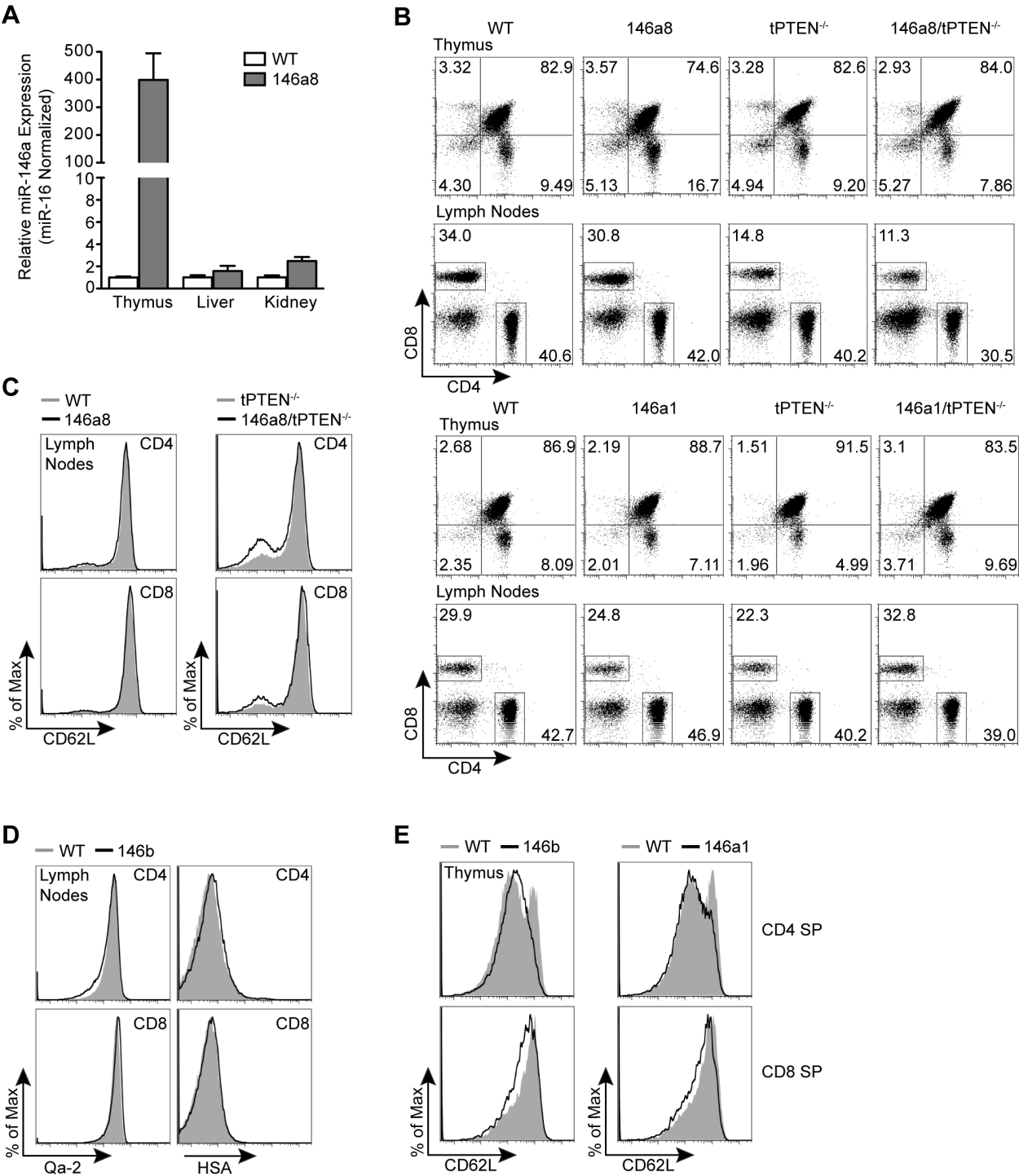


Figure S2. T cell maturation is largely normal in *mir-146a* and *mir-146b* transgenic mice. (A) miR-146a expression in *146a8* transgenic mice in the thymus versus the liver and kidney. Transgene expression is not detected in non-lymphoid tissues. Fold change is relative to miR-146a expression in WT tissue. (B) CD4 versus CD8 flow cytometric analysis of T cell populations in the thymus and lymph nodes of *mir-146a* transgenic lines *146a8* and *146a1* compared to littermate controls on both WT and tPTEN^{-/-} backgrounds (C57BL/6 background). Mice were 11-weeks-old and 12.5-weeks-old respectively. (C) Flow cytometric analysis of CD62L (L-selection) surface expression in lymph node T cells from 7-week-old *146a8* transgenic and littermate control mice on WT and tPTEN^{-/-} backgrounds. Data are representative of 2 independent experiments. (D) Flow cytometric analysis of maturation markers Qa-2 and HSA in lymph node T cells of 8-week-old littermate *146b* transgenic and WT mice. Data are representative of 2 independent experiments. (E) Flow cytometric analysis of CD62L surface expression in SP thymocytes from 8-week-old *146b* and *146a1* transgenic mice and WT littermate controls. Data are representative of 2 independent experiments.

Figure S3

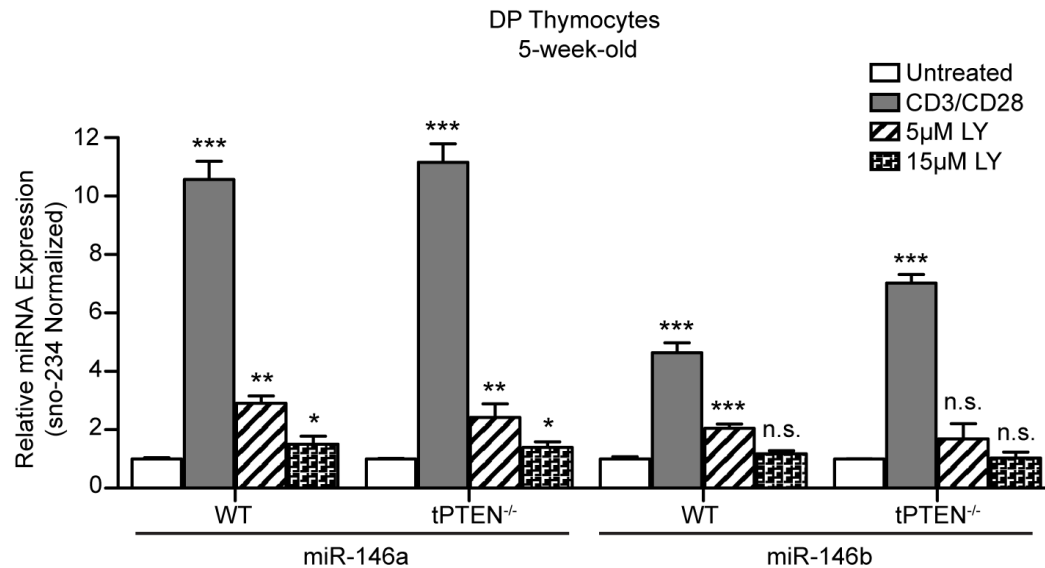


Figure S3. Akt-dependent upregulation of miR-146a and miR-146b in TCR-stimulated DP thymocytes is also evident with sno-234 normalization. qRT-PCR analysis of miR-146a and miR-146b expression levels in FACS sorted DP thymocytes from 5-week-old mice as in Figure 5A. Thymocytes were left unstimulated or stimulated with anti-CD3/CD28 antibodies for 16 hours. Where indicated, thymocytes were pre-treated with 5µM or 15µM LY294002 (LY) for 1 hour prior to anti-CD3/CD28 stimulation. Fold change is relative to untreated samples and normalization was performed with sno-234 instead of miR-16. Data are presented as mean $\pm$ SD and are representative of 2 independent experiments. Statistics were calculated by Student's t-test: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. not significant.

Figure S4

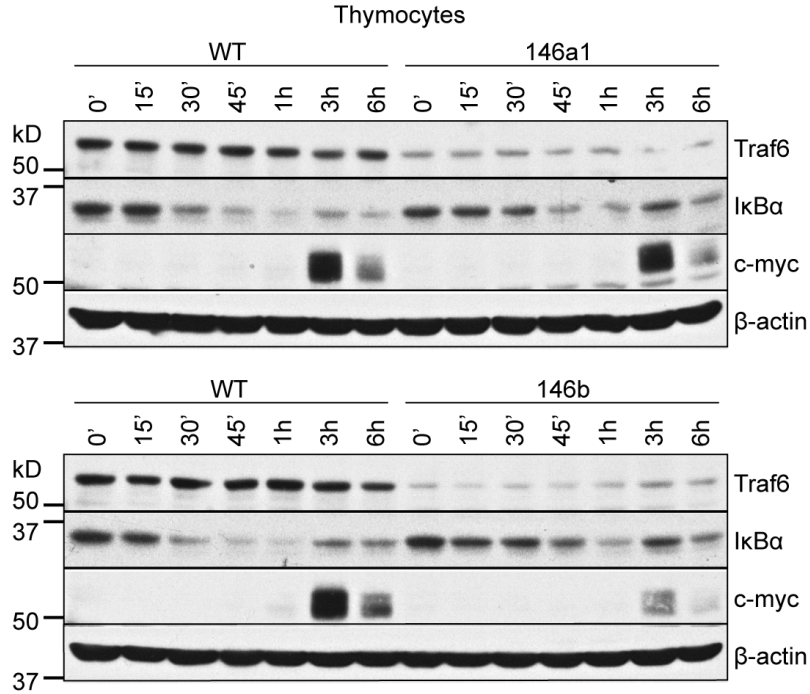


Figure S4. Attenuated IκBα degradation and c-myc induction in TCR-stimulated *mir-146a* and *mir-146b* transgenic thymocytes. Western blot analysis of Traf6 expression, IκBα degradation and c-myc induction in TCR-stimulated total thymocytes from 10-week-old *146a1* and *146b* transgenic mice and WT littermate controls. Thymocytes were stimulated with PMA and Ionomycin for the indicated times. Data are representative of 3 or more independent experiments.

Figure S5

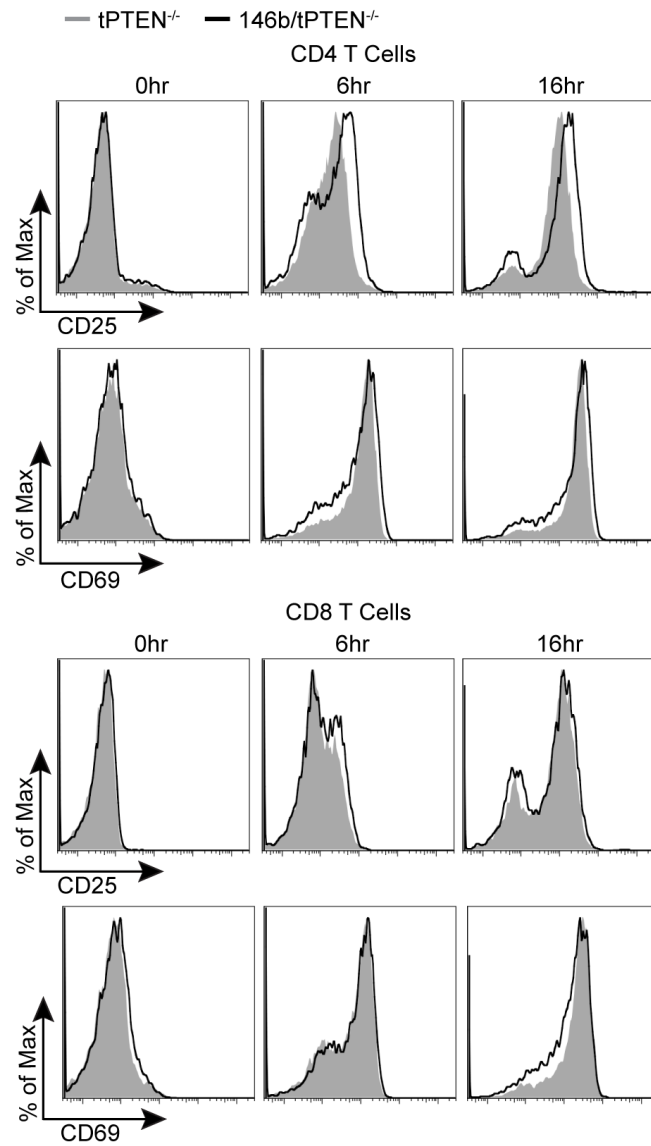


Figure S5. *mir-146b* transgenic T cells upregulate activation markers normally upon TCR-stimulation. Flow cytometric analysis of CD25 (IL-2R α) and CD69 surface expression on 7-week-old *146b/tPTEN*^{-/-} and tPTEN^{-/-} littermate control T cells stimulated with anti-CD3/CD28 antibodies for the indicated times.

Figure S6

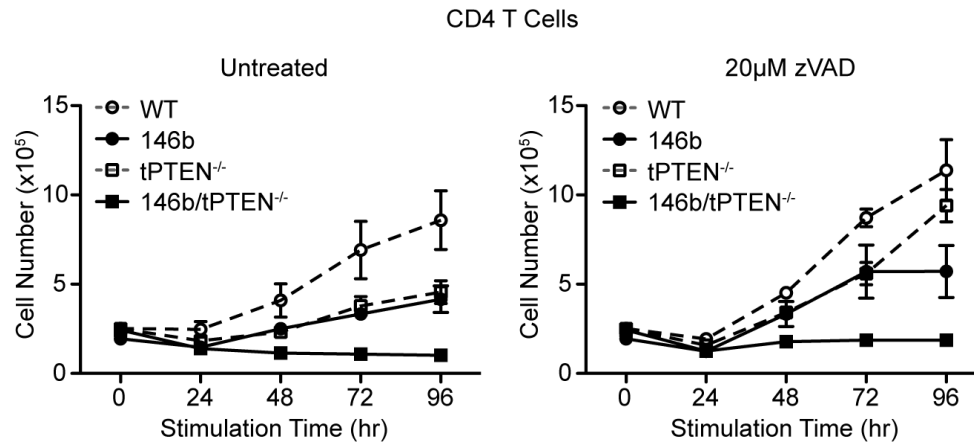


Figure S6. Inhibition of apoptosis by zVAD-FMK does not rescue the *mir-146b* transgenic CD4 T cell expansion defect. TCR-mediated expansion of CD4 and CD8 T cells from 6 to 10-week-old *146b* transgenic mice compared to littermate controls on WT and tPTEN^{-/-} backgrounds. Total T cells were left untreated or treated with 20µM zVAD-FMK concurrently with anti-CD3/CD28 antibody stimulation. Triplicate T cell samples were collected at each time point and total live T cell numbers were determined by Trypan blue exclusion. CD4 and CD8 numbers were calculated based on flow cytometric analysis at each time point.

Figure S7

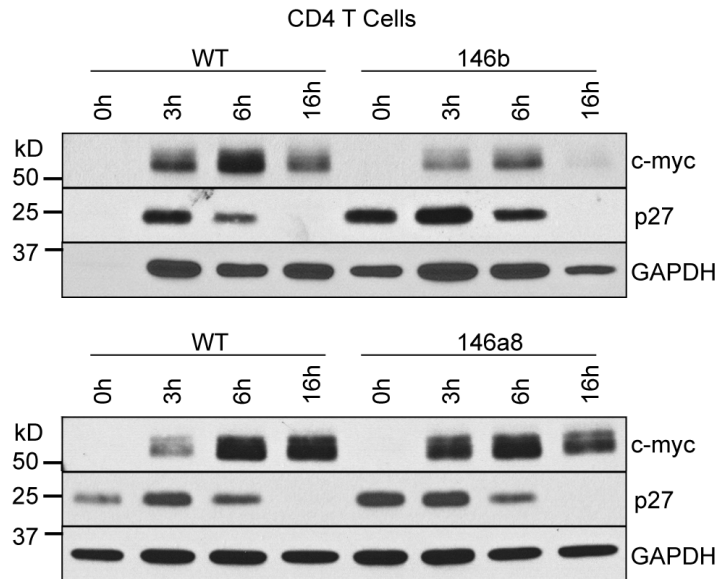


Figure S7. Over-expression of miR-146b, but not miR-146a, in PTEN-sufficient T cells inhibits TCR-mediated c-myc induction. Western blot analysis of c-myc induction and p27 levels in purified CD4 T cells from 7 to 9-week-old *146b* and *146a8* transgenic mice and WT littermate controls stimulated with anti-CD3/CD28 antibodies for the indicated times. Data are representative of 2 independent experiments.

Figure S8

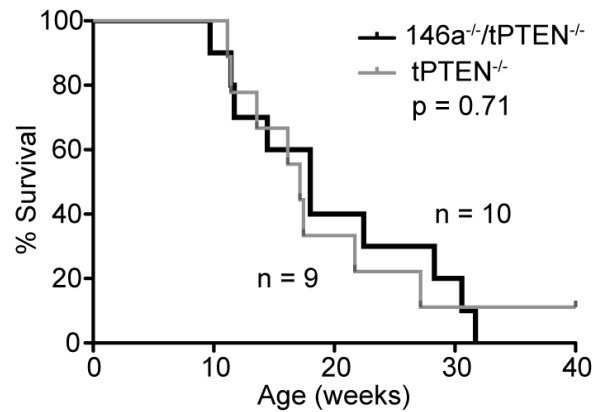


Figure S8. *mir-146a* deletion does not accelerate $tPTEN^{-/-}$ lymphomagenesis.

Kaplan-meyer curves for *mir-146a*-deficient mice (described previously¹²⁴) crossed with $tPTEN^{-/-}$ mice on the C57BL/6 background. Statistical significance was calculated using the Gehan-Breslow-Wilcoxon method.

Concluding Remarks and Outlook

T cell development in the thymus is tightly regulated and highly selective to ensure the contribution of only the most fit T cells to the adaptive immune system. The fate of a developing thymocyte is determined by the life or death signals it receives at various checkpoints throughout this process. Thymocytes with T cell receptors that react too strongly to self are fated to undergo apoptosis to prevent the contribution of autoreactive T cells to the mature T cell repertoire. The work presented here in Chapters 2 and 3 adds to our knowledge of the apoptotic mechanisms mediating this process of thymocyte negative selection. The Nr4a nuclear receptors have long been thought to promote apoptosis during negative selection, but the mechanism of their action remains unclear. Here, through the characterization of Bcl-2 BH3 mutant transgenic mice, we provide *in vivo* support for the proposed Nr4a/Bcl-2 mitochondrial killing mechanism. We also tease out some of the regulation governing Nr4a's translocation to the mitochondria. Further studies are needed to determine the relative contribution of this Nr4a/Bcl-2-mediated apoptotic pathway versus Bim-mediated apoptosis during negative selection. In our Bcl-2 BH3 mutant transgenic mice, negative selection is rescued by both inhibition of Bim (mediated by the BH4 domain) and inhibition of the BH3 domain pro-apoptotic function. A knock-in Bcl-2 BH3 mutant mouse could be used to assess the impact of disrupting the Nr4a/Bcl-2 mechanism while leaving Bim-mediated apoptosis intact. One caveat of this approach is the possibility of redundancy between Bcl-2 and other family members, such as Bcl2A1. Alternatively, one could modify the Bcl-2 BH3 mutant transgene described here to relieve inhibition on Bim through mutation of the BH4 domain. In this over-expression system, the Nr4a receptors would be preferentially bound by the abundant transgenic protein. It is possible that the Nr4a receptors also promote negative selection through a nuclear killing mechanism, which could confound these results. While more difficult, it would also be interesting to evaluate negative selection in mice expressing a Nor-1 protein lacking mitochondrial translocation ability on a Nur77/Nurr1-deficient background.

In Chapter 2 of this work, we found that Bcl-2 BH3 mutant transgenic mice, in contrast to wild-type Bcl-2 transgenic controls, develop multi-organ autoimmune disease and die prematurely. This phenotype is likely the consequence of a more pronounced defect in negative selection due to BH3 mutation and provides new evidence for a crucial role for central tolerance in the prevention of autoimmune disease. It is possible, however, that use of the CD4 promoter to drive BH3 mutant transgene expression, rather than the Lck promoter used for the wild-type transgene, is somehow responsible for the difference in phenotype between these mice. Downregulation of the Bcl-2 BH3 mutant transgene in the peripheral CD8 T cell compartment might accelerate the appearance of autoimmunity due to relief of Bcl-2's inhibitory effects on T cell proliferation. This could be clarified by comparison of Bcl-2 BH3 mutant and wild-type Bcl-2 transgenes driven by the same promoter, preferably Lck to promote expression in both CD4 and CD8 SP cells. Lines exhibiting proper downregulation of transgene expression in the periphery should be selected to allow normal mature T cell proliferation.

Finally, in Chapter 4 of this work we investigated the consequences of aberrant survival signals, rather than absent death signals, on the fate of DP thymocytes. Similar to previous reports, we found that loss of PTEN in DP thymocytes results in the formation of premalignancies that eventually progress to mature T cell lymphomas. We identified two miRNAs, miR-146a and miR-146b that are upregulated in DP premalignancies as a defense against malignant transformation. These miRNAs negatively regulate TCR signals that are critical for c-myc upregulation and progression to malignancy. An array of recent studies has similarly implicated miR-146a in having tumor suppressive function in multiple cancer types. Thus, future studies could test the therapeutic potential of these miRNAs in T cell and other malignancies. The anti-tumor effects of local or systemic delivery of these miRNAs in mouse models should be evaluated.

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Appendix A

Upregulation of the miR-183/96/182 microRNA cluster during premalignancy has a neutral impact on PTEN-deficient lymphomagenesis

Introduction

Phosphatase and Tensin Homolog (PTEN) is one of the most frequently inactivated tumor suppressors in human cancers, including T cell leukemia and lymphoma^{47,135}. To better understand how T cell malignancies form in the absence of PTEN, we and others have carried out studies in a T cell-specific PTEN-deficient mouse model (tPTEN^{-/-}). tPTEN^{-/-} mice spontaneously develop mature T cell tumors in the lymph nodes and spleen^{49,51}. Tumor development is preceded by premalignancies in the thymus, characterized by DP thymocyte blasting, increased signaling through the Akt pathway and acquisition of *TCRa/c-myc* chromosomal translocations^{50,51,136}. Here, we sought to identify factors that delay progression of these premalignancies to tumors in the periphery. As discussed in Chapter 4, we identified several miRNAs that are upregulated specifically in DP thymocytes during the premalignant period. Amongst the most highly upregulated miRNAs were miR-146a, miR-96 and miR-182, two of which are part of the miR-183/96/182 cluster. miR-183 was subsequently shown to be upregulated as well, consistent with miR-96, miR-182 and miR-183 being derived from a single transcript. Through transgenic over-expression of miR-146a and its family member miR-146b in T cells, we found that miR-146a/b can significantly delay tPTEN^{-/-} lymphomagenesis through negative regulation of TCR signaling necessary for c-myc upregulation (Chapter 4)¹³⁶. Here, we sought to investigate whether the miRNAs of the miR-183/96/182 cluster could similarly act to regulate tumorigenesis in tPTEN^{-/-} mice.

Results and Discussion

To investigate the role of miR-183/96/182 cluster upregulation in the development of PTEN-deficient lymphomas, we created T cell-specific miR-183/96/182 transgenic mice. Similar to the miR-146a and miR-146b transgenic mice described in Chapter 4, the *miR-183/96/182* transgene was expressed under the control of the CD4 regulatory elements.^{66,104,136} The transgenic mice were generated on the C57BL/6 background and three “miR-Cluster” founder lines were used for analysis, miR-Cluster A, B and D.

Expression levels of miR-183, miR-96 and miR-182 in thymocytes and mature T cells of miR-Cluster D and A transgenic mice were examined by qRT-PCR. As the transgene is driven by the CD4 promoter, low miRNA expression levels were observed in CD4⁻ CD8⁻ double negative (DN) (Figure 1). Some background expression of the Cluster A transgene was observed in these cells. Cluster transgenic CD4⁺ CD8⁺ double positive (DP) thymocytes expressed very high levels of all three miRNAs compared to wild-type controls. The greatest fold upregulation was observed for miR-96, which is barely expressed in wild-type cells. miR-183, miR-96 and miR-182 were also upregulated in CD4 single positive thymocytes (CD4 SP) and expression was maintained in the mature lymph node T cell population. However, expression levels in CD4 SP and mature T cells were not as high as those observed in DP thymocytes. The pattern of miRNA expression between the Cluster D and Cluster A lines was very comparable, except for the background expression in the Cluster A DN population. While the miR-Cluster B expression levels were not examined by qRT-PCR, based on

genomic DNA amplification by genotyping PCR, the Cluster B transgene is expressed at lower levels than the Cluster D and A transgenes (data not shown).

To determine whether over-expression of the miR-183/96/182 cluster in thymocytes impacts PTEN-deficient lymphomagenesis, we crossed the Cluster transgenic mice to tPTEN^{-/-} mice. Survival curves were generated to assess the rate of lymphomagenesis. For all three founder lines examined, miR-183/96/182 transgene expression neither extended nor shortened the lifespan of tPTEN^{-/-} mice (Figure 2). Thus, upregulation of this miRNA cluster during premalignancy appears to play a neutral role in the development of PTEN-deficient lymphomas.

Upregulation of the miR-183/96/182 cluster during premalignancy likely occurs as a byproduct of amplified signaling in the DP cells. In a panel of human acute T cell lymphoblastic leukemia (T-ALL) cell lines, we find that miR-183/96/182 are abundantly expressed in PTEN null lines that are dependent on Akt signaling for survival (Figure 3)¹³⁵. Conversely, expression is undetectable in PTEN wild-type, Notch-dependent cell lines. These data suggest that miR-183/96/182 might be upregulated downstream of the enhanced Akt signaling observed in premalignant tPTEN^{-/-} thymocytes. As miR-183/96/182 upregulation also correlates with c-myc upregulation (Chapter 4), it is also possible that c-myc could drive miR-183/96/182 expression. However, we find that c-myc activation in the thymus of Rosa26-mycER mice (Figure 4A) does not induce expression of these miRNAs (Figure 4B), suggesting they are not regulated by c-myc.

While upregulation of the miR-183/96/182 cluster results in a net neutral phenotype in our model, prior studies suggest that these miRNAs could play an important role in amplifying Akt signaling by targeting Foxo proteins. In breast cancer cells, repression of Foxo1 by miR-96 and miR-182 supports cell viability and proliferation¹³⁷. miR-182 and miR-183 have also been shown to target Foxo3, promoting tumor cell survival and migration^{138,139}. In normal T cells, miR-182 is upregulated downstream of IL-2 signaling and targets Foxo1 to promote proliferation of T cells post-activation¹⁴⁰. Consistent with these findings, we find that Foxo1 levels are lower in tPTEN^{-/-} thymocytes and mature T cells from premalignant and malignant mice compared to normal wild-type controls (Figure 5). Increased Foxo3 phosphorylation (which promotes its inactivation) has also been reported in premalignant DP thymocytes⁵¹. Downregulation of the cell cycle inhibitor p27, a Foxo target gene, is evident in both thymocytes and mature T cells⁵¹. Whether Foxo and p27 levels are also decreased in younger, normal tPTEN^{-/-} mice has yet to be tested.

Materials and Methods

Mice

The miR-183/96/182 transgene was created by restriction digest of mouse C57BL/6J BAC RP23-1D15 (30040255-30252147, CHORI) with KpnI. A ~9kb fragment was subsequently cloned into pBluescript SK+ (Stratagene) at a KpnI site. The insert was then shuttled to pCI (Promega) at KpnI and cut out with XhoI and Sall to be cloned into pcDNA3.1 (Invitrogen) at an XhoI site (Sall/XhoI ligation destroyed the C-terminal XhoI cut site). The insert was then cut out with XhoI and HpaI for ligation behind the CD4 promoter of pTG4^{66,104}. The pTG4 construct was modified to include the *cd4* locus control region (LCR). PCR amplification of the 1kb LCR sequence from C57BL/6

genomic DNA was performed with the following primers: LCR forward GACATCGATAGCTAGCACACGCCGGTAAGCCCATTCACACGC, LCR reverse GACATCGATGCGGTACCGATCCCAACCAAACTGCGGCCCTTTCA. The LCR was added to the 5' end of the CD4 silencer at a *Cl*I restriction site. Transgenic founders were identified by PCR genotyping with the following primers: HGH forward GACACAAACTCACACAACGATGACGC, HGH reverse ATGCCTGGAATCCCAACAACTCGG. After crossing, genotyping was performed with miR-Cluster-forward CTTGCCAACTATGGTGTAAAGTGC and miR-Cluster-reverse GCAAGAGGCCAACACTCTCC.

Characterization of the *Lck*-Cre *Pten*^{fl/fl} (*tPTEN*^{-/-}) mice has previously been reported^{49,51}. Analyses were performed on a C57BL/6 background (backcrossed more than 10 generations), with the exception of the *Foxo1* qRT-PCR experiment, which was performed on a mixed 129Sv/J x CBA x C57BL/6 background.

Rosa26-mycER mouse experiments were performed in collaboration with Daniel Garcia in Gerard Evan's lab. C-myc activation by tamoxifen treatment of *Rosa26*-mycER mice was described previously. Here, *Rosa26*-mycER homozygous mice of a mixed genetic background (*R26*^{MER/MER}) were injected daily with either tamoxifen (1mg/20g body mass), dissolved in peanut oil, or oil alone intraperitoneally for 3 days.

Quantitative Real-Time RT-PCR

Lymphoid organs were isolated and cells dissociated through 40µm cell strainers and red blood cell lysis was performed. Thymocytes were stained with fluorochrome-conjugated antibodies (eBioscience, San Diego, CA, USA and BD Biosciences, San Jose, CA, USA) in 1% fetal bovine serum (FBS) in PBS and then transferred to 50% FBS RPMI media. DN, DP and CD4 SP thymocyte populations were collected by cell sorting with the Cytopeia (Seattle, WA, USA) Influx Sorter or the DAKO-Cytomation (Carpinteria, CA, USA) MoFlo High Speed Sorter. Mature T cells were purified by negative selection columns from the lymph nodes and spleen (T Cell Enrichment Columns, R&D). Total RNA was extracted from primary lymphoid cells or leukemia cells lines with a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Taqman MicroRNA Assays (Applied Biosystems, Life Technologies, Grand Island, NY, USA) specific to each miRNA were used for miRNA quantitation according to the manufacturer's protocol. For mRNA quantitation, reverse transcription was performed using SuperScript III with Oligo(dT) primers (Invitrogen, Life Technologies). SYBR Green (Invitrogen) real-time PCR was performed using the following primers: GAPDH-forward ATCTTCTTGTGCAGTGCCAGCCTCGTCCCG, GAPDH-reverse AGTTGAGGTCAATGAAGGGGTCGTTGATGG, mouse c-myc-forward GCCCCTAGTGCTGCATGA, mouse c-myc-reverse AGAAGATGAGGAAGAAATTGATGTGG, human c-myc forward CTTCTCTCCGTCCTCGGATTCT, human c-myc reverse GAAGGTGATCCAGACTCTGACCTT, *Foxo1*-forward GGATGGTGAAGAGCGTGCCCTA and *Foxo1*-reverse GGGACAGATTGTGGCGAATTGA.

Figure 1

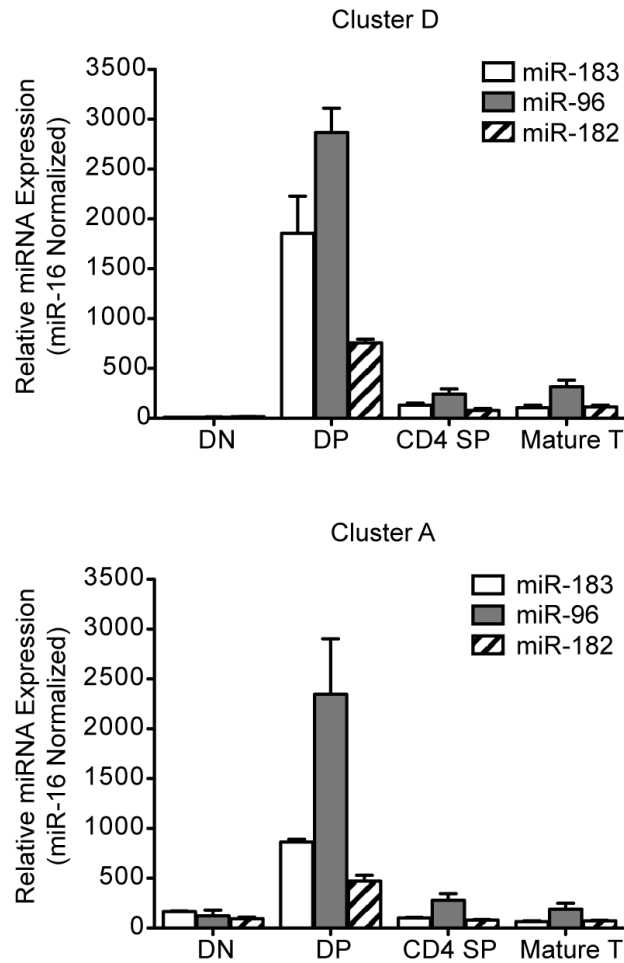


Figure 1. miR-183/96/182 cluster expression in T cell subsets of miR-Cluster transgenic mice. qRT-PCR analysis of miR-183, miR-96 and miR-182 miRNA in Cluster D and Cluster A transgenic mice. Fold change is relative to wild-type littermate controls. miRNA expression was normalized to miR-16.

Figure 2

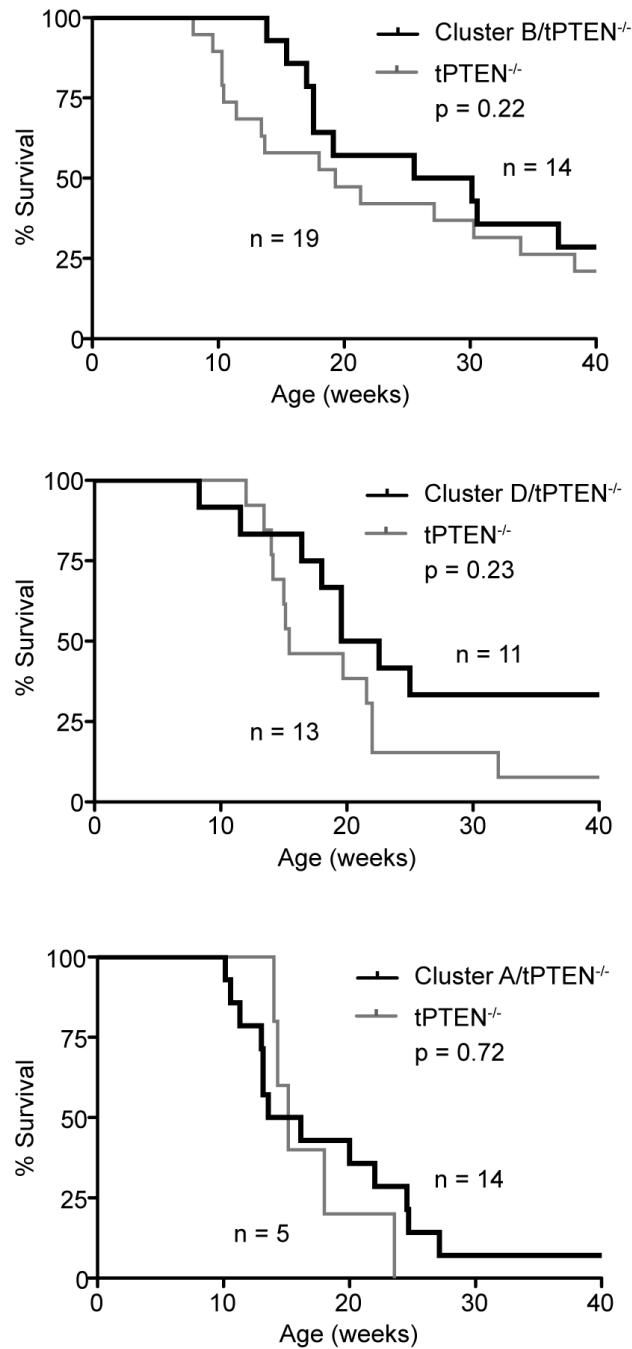


Figure 2. T cell-specific transgenic expression of the miR-183/96/182 cluster does not impact the survival of PTEN-deficient mice. Kaplan-meyer curves for the Cluster B, D and A transgenic lines crossed to $tPTEN^{-/-}$ mice on the C57BL/6 background. Statistical significance was calculated using the Gehan-Breslow-Wilcoxon method.

Figure 3

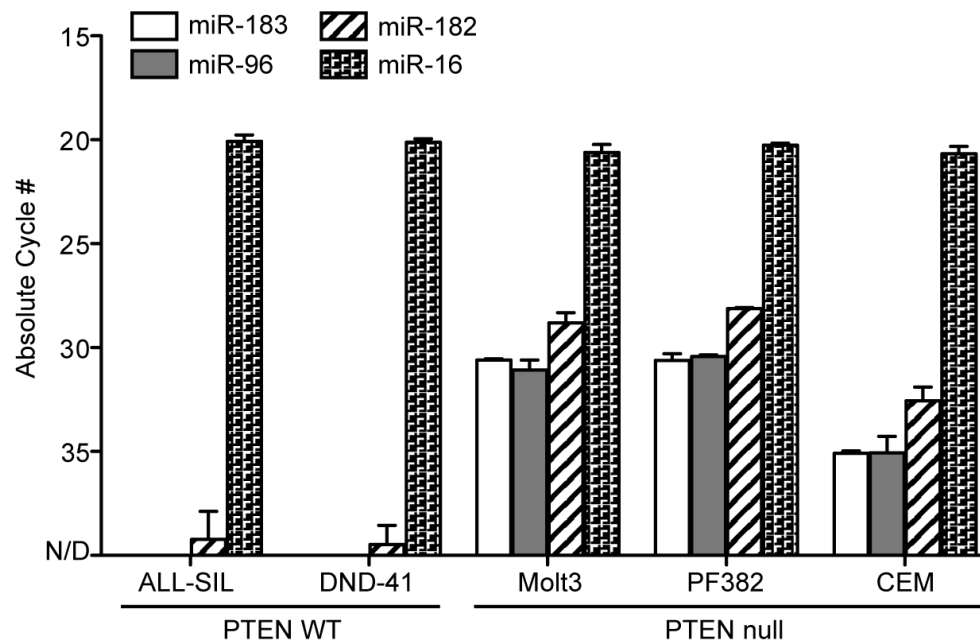


Figure 3. The miR-183/96/182 cluster is highly expressed in PTEN null, Akt-dependent T cell leukemia cell lines. qRT-PCR analysis of miR-183, miR-96 and miR-182 expression in PTEN WT versus PTEN null acute T cell lymphoblastic leukemia (T-ALL) cell lines. PTEN WT cell lines are dependent on Notch-signaling for survival whereas PTEN null cell lines require Akt signaling¹³⁵. miRNA expression is graphed as absolute qRT-PCR cycle number, with a cycle number of 40 marking the limit of detection (N/D = not detectable). miR-16, a miRNA that is very abundant in T cells and is similarly expressed in all the T-ALL cell lines, serves as a reference for the abundance of miR-183/96/182 cluster miRNAs.

Figure 4

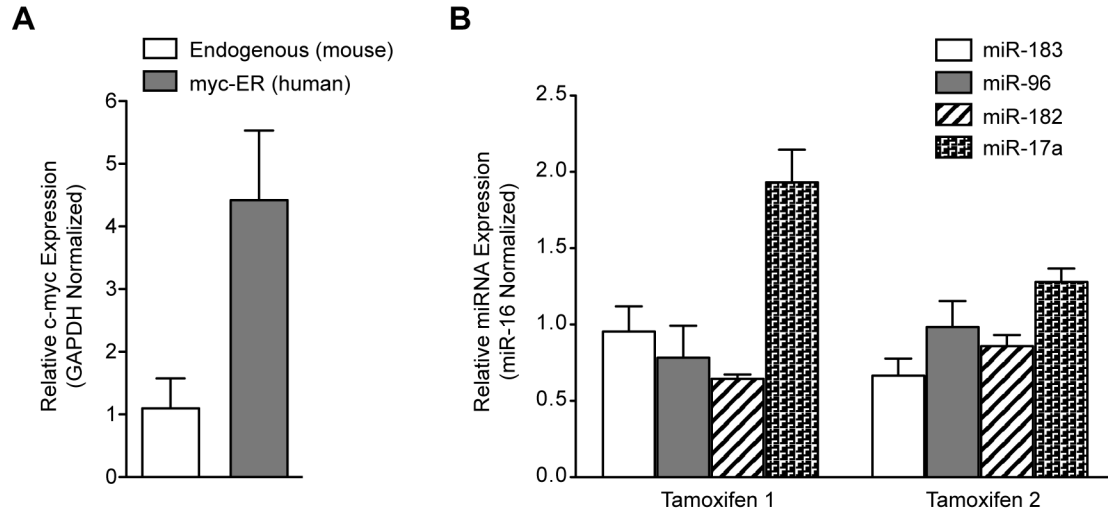


Figure 4. C-myc activation in Rosa26-mycER mice does not induce miR-183/96/182 upregulation in the thymus. (A) qRT-PCR analysis of human mycER expression versus endogenous c-myc expression in total thymocytes from homozygous Rosa26-mycER mice ($R26^{MER/MER}$). Fold change is relative to the level of endogenous mouse c-myc expression. Human mycER expression (detected by human-specific primers) is approximately 3 times higher than endogenous c-myc expression (detected by mouse c-myc primers). Human/mouse c-myc expression was normalized to GAPDH. (B) $R26^{MER/MER}$ mice were injected with tamoxifen or oil daily for 3 days to activate mycER. miR-183/96/182 expression was assessed by qRT-PCR. Fold change in miRNA expression for two tamoxifen-treated mice relative to oil-treated controls is shown. As a positive control, we observed induction of miR-17a expression with mycER activation.

Figure 5

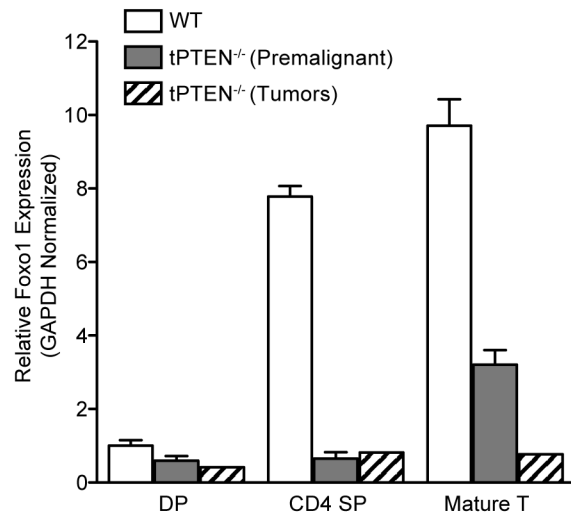


Figure 5. Foxo1 is downregulated in thymocytes and mature T cells from premalignant and tumor-bearing tPTEN^{-/-} mice. qRT-PCR analysis of Foxo1 expression in T cell populations from 9-week-old tPTEN^{-/-} premalignant and tumor-bearing mice compared to a wild-type (WT) littermate control. Fold change is relative to WT DP. Foxo1 expression was normalized to GAPDH.