Lymphoma development in *Bax* transgenic mice is inhibited by *Bcl-2* and associated with chromosomal instability

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Abstract

Bax is a *Bcl-2* family member that promotes apoptosis but has paradoxical effects on lymphoma development in *p53*-deficient mice. To better understand the mechanism of *Bax*-induced lymphoma development, the effect of *Bax* levels, *p53* status and *Bcl-2* coexpression on lymphoma development were determined. In addition, DNA content and cytogenetics were performed on young (pre-malignant) Lck-*Bax* mice as measures of genetic instability. *Bax* promoted lymphoma development in *p53*-deficient mice in a dose-dependent manner. *Bax* expression also led to lymphoma development in both *p53* +/– and +/+ animals. Ploidy analysis in mice prior to the onset of overt thymic lymphomas demonstrated that Lck-*Bax* transgenic mice were more likely to be aneuploid and demonstrate increased chromosome instability. With tumor progression, aneuploidy increased and *Bax* expression was maintained. Importantly, coexpression of *Bcl-2* delayed lymphoma development in Lck-*Bax* transgenic mice. These data support a model in which increased sensitivity to apoptosis leads directly to chromosome instability in developing T cells and may explain a number of paradoxical observations regarding *Bcl-2* family members and the regulation of cancer. *Cell Death and Differentiation* (2003) 10, 740–748. doi:10.1038/sj.cdd.4401233

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Abbreviations: PCR, polymerase chain reaction; CV, coefficient of variation; CIN, chromosomal instability.

Introduction

The *Bcl-2* family contains a number of related genes that are critical regulators of apoptosis.¹ The family can be divided into those that inhibit (*Bcl-2*, *Bcl-x*, etc.) and those that promote cell death. Those that promote apoptosis can be further characterized as multidomain members (*Bax*, *Bak*, *Bok*) and BH3-only family members based on the extent of homology with *Bcl-2*.² The *Bcl-2* family appears to regulate apoptosis through effects on mitochondria. Antiapoptotic members preserve mitochondrial function and prevent the release of mitochondrial proteins such as cytochrome *c*, *Smac/Diablo* and *AIF*, thereby preventing caspase activation and DNA degradation, two hallmarks of apoptosis.³ The ability of *Bcl-2* to act upstream of caspases and preserve mitochondrial function may contribute to its more potent oncogenic activity relative to other antiapoptotic genes that act downstream such as by inhibition of caspases.⁴ The mechanism by which *Bcl-2* prevents the release of mitochondrial components remains controversial, but *Bcl-2* appears to prevent the formation or opening of a large mitochondria channel that involves proapoptotic family members and/or the voltage-dependent anion channel.³

Recent studies have demonstrated that the BH3-only *Bcl-2* family members act upstream of the multidomain members *Bak* or *Bax* to disrupt mitochondria function and release cytochrome *c*.^{5,6} Furthermore, the main function of *Bcl-2* or *Bcl-x* may be to prevent BH3-only family members from 'activating' *Bax* or *Bak* and releasing proapoptotic mitochondria components.⁷ Regardless of the precise mechanism, *Bcl-2* is clearly an important mediator of oncogenesis. This is perhaps best illustrated by the involvement of *Bcl-2* in the t(14:18) translocation follicular B-cell lymphoma^{8–10} and has been further demonstrated in transgenic mice.^{11,12} Furthermore, a number of findings implicate the loss of function of the proapoptotic family member *Bax* in oncogenesis.^{13–18} The *Bax* gene is inactivated in human colon cancer of the microsatellite mutator phenotype¹⁹ and *Bax*-deficient cells appear resistant to cell death following activation of the Trail receptor pathway.²⁰

Thus, a number of lines of evidence demonstrate that decreased apoptosis because of alterations in the *Bcl-2* family can promote oncogenesis. However, other studies demonstrate paradoxical effects of *Bcl-2* family members on oncogenesis. For example, expression of *Bcl-2* delays mammary tumor formation in dimethylbenz(a)anthracene treated mice²¹ and hepatocellular carcinoma in c-myc transgenic mice.²² Studies of human colon cancer^{23–27} and breast cancer²⁸ have demonstrated that *Bcl-2* expression is associated with improved survival. For *Bax*, increased expression in transgenic mice is associated with increased apoptosis and a paradoxical acceleration in lymphoma

development in *p53*^{-/-} mice.²⁹ In human cancer, increased *Bax* expression has been associated with an increase risk of relapse in childhood acute lymphocytic leukemia³⁰ and decreased survival in diffuse large B-cell lymphoma.³¹ Therefore, the outcome of *Bcl-2* family expression on cancer is complex and may involve regulation of cellular processes other than apoptosis. Alterations in cell proliferation have been proposed to account for the paradoxical effect of *Bcl-2* on oncogenesis.^{21,22} However, to date these studies are largely correlative and fall short of proving causation.

The current study demonstrates that aneuploidy is an early and frequent result of expression of *Bax* and provides an alternative explanation for the paradoxical effects of *Bcl-2* family members on oncogenesis. Specifically, *Bcl-2* family members may influence oncogenesis through regulation of chromosome stability. This complements and extends pre-

vious data demonstrating that chromosome instability is frequently associated with increased apoptosis.

Results

Tumor development in Lck-Bax transgenic mice

Lck-Bax transgenic mice accelerate lymphoma development in *p53*-deficient mice. These paradoxical findings were true for two transgenic lines (1 and 38) derived from distinct vectors with differences in the 3' untranslated region of the cDNA.²⁹ This difference has allowed us to determine if the effects of *Bax* on lymphoma development are additive without requiring homozygous expression of a single transgene. The rate of spontaneous lymphoma development in Lck-Bax38/1 double transgenic *p53*^{-/-} mice was determined. When compared to the *p53*-deficient mice alone, the Lck-Bax38/1 double transgenic animals developed lymphoma at an accelerated rate (Figure 1). These differences were also true when they were compared only to littermate controls that were included in the study (data not shown). Furthermore, when compared to our previous results with the single transgenics,²⁹ the Lck-Lck-Bax38/1 double transgenics developed lymphoma at a faster rate than either Lck-Bax1 or Lck-Bax38 alone demonstrating quantitative effects of *Bax* on tumor development (Figure 1).

Lck-Bax38/1 promotes lymphoma in *p53* +/- and +/- mice

Although Lck-Bax38 or Lck-Bax1 accelerated tumor development in *p53*^{-/-} mice, they were not sufficient to accelerate tumor development in *p53* +/- mice (data not shown). However, a number of Lck-Bax38/1 double transgenic mice that were not *p53* deficient were found to be sick and many died from thymic tumors at a relatively young age (<1 year). Therefore, the rate of tumor development in Lck-Bax38/1 double transgenic mice with functional *p53* was determined. Both Lck-Bax38/1-*p53* +/- or *p53* +/- mice had markedly decreased survival (Figure 2a,b) secondary to an increase in

Figure 1 Additive effect of Bax on tumor development in *p53*-deficient mice. Kaplan-Meier survival plot of *p53*^{-/-} mice (◆), and *p53*^{-/-} mice expressing Lck-Bax1 (■), Lck-Bax38 (□) or both Lck-Bax38 and Lck-Bax1 (◇) are shown. Lck-Bax1 ($P=0.0211$), Lck-Bax38 ($P<0.0001$) and Lck-Bax38/1 ($P<0.0001$) all had significantly decreased survival relative to control (*p53*^{-/-}) mice. Furthermore, the Lck-Bax38/1 had significantly decreased survival when compared to Lck-Bax1 ($P=0.0027$) and Lck-Bax38 ($P=0.0472$) alone. The data from the single transgenics have been previously published²⁹

Figure 2 Lck-Bax38/1 double transgenic mice develop tumors independent of *p53* deficiency. Lck-Bax38/1 transgenic mice (◆) on either a *p53* +/- (a) or +/- (b) background were followed for overall survival and compared with negative littermates that were matched for *p53* status (◇). Lck-Bax38/1 significantly decreased survival in *p53* +/- (a) and *p53* +/- mice (b) relative to control animals that all survived. The time at which the control mice were censored is indicated by an open diamond (◇). Control mice were killed when they reached 1 year of age as described in Materials and Methods. (c) Lck-Bax38/1 tumor formation in *p53* +/- (◆) and *p53* +/- mice (○) mice were not significantly different ($P=0.7825$)

Table 1 Increased coefficient of variation of diploid cells in Lck-Bax mice

	Neg	Bax1	Bax38	Bax38/1
CV mean	1.687	2.294	2.224	2.661
P-value (versus Neg)		0.001	0.005	0.0001
STD	0.472	0.547	0.618	0.318
N	21	15	15	14

thymic lymphomas. Surprisingly, the rate of tumor development was similar between *p53* +/+ and *p53* +/- mice, suggesting that loss of function of *p53* may not be the rate-limiting step in lymphoma development of Lck-Bax38/1 mice that contain functional *p53* (+/- or +/+) (Figure 2c). These results demonstrate that overexpression of *Bax* promotes thymic lymphoma development in mice with intact *p53*.

Aneuploidy in Lck-Bax-*p53* -/- mice

Lck-Bax transgenic mice demonstrate increased thymocyte proliferation which may explain the accelerated lymphoma development.²⁹ However, the total number of cycling cells in Lck-Bax mice was not increased because of lymphopenia from the increased apoptosis.²⁹ In the process of performing these studies on cell proliferation, the G0/G1 diploid peak demonstrated an increased coefficient of variation (CV) in Lck-Bax transgenic mice (Table 1). One explanation for the widened peak may be altered DNA content because of increased chromosomal instability. Chromosomal instability is observed in many types of tumors and has been described as an enabling characteristic of tumor cells allowing them to acquire the many genetic changes necessary for oncogenesis.³² If high levels of *Bax* promote chromosomal instability, this would provide an alternative explanation for the accelerated tumor formation in Lck-Bax mice. To determine if *Bax* promotes chromosomal instability, DNA content analysis was performed on young Lck-Bax-*p53* -/- mice using propidium iodide staining. Lck-Bax-*p53* -/- mice frequently demonstrated more than one near diploid peak, suggesting that an aneuploid population is present in these mice (Figure 3a). Of note, using this relatively insensitive measure of aneuploidy, ~56% of young Lck-Bax mice were found to be aneuploid (mice with overt thymic lymphomas were excluded) while only 11% of control *p53*-deficient mice were aneuploid (Table 2). Importantly, the development of an aneuploid population appears to precede overt tumor development based on the fact they are frequently observed in animals with reduced cellularity (Figure 3b). Cytogenetics performed on the thymocytes from a Lck-Bax mouse confirmed the presence of an aneuploid population in one of these animals (Figure 3c).

Aneuploidy in Lck-Bax-*p53* control mice

Given that *Bax* expression was able to induce thymic lymphomas independent of *p53* (Figure 2), Lck-Bax38/1 transgenic animals that were *p53* +/- or +/+ were also examined for DNA content using propidium iodide staining. For these animals, aneuploidy was infrequently detected. This may be because of elimination of aneuploid populations by

functional *p53* at a point prior to their expansion and detection using DNA content analysis. Nonetheless, in some *p53* +/- and +/+ animals, aneuploid populations were identified and this was seen in animals with a very small thymus (less than 2 million cells) (Figure 4). This demonstrates that aneuploidy is seen in Lck-Bax transgenic mice with functional *p53*.

Aneuploidy and Bax expression in tumors from Lck-Bax transgenic mice

If *Bax* expression directly causes lymphoma development and chromosome instability, expression of *Bax* would be maintained in tumors from these animals. To test this prediction, lysates of thymic lymphomas from control and Lck-Bax transgenic mice were probed for *Bax* expression. As expected, *Bax* expression was increased in tumors from Lck-Bax-positive mice relative to control thymus or tumors from *p53*-deficient mice with or without *Bcl-2* expression (Figure 5a). In addition, the extent and diversity of aneuploid cells is increased in the tumors from Lck-Bax transgenic mice (Figure 5b). These data support a direct and intrinsic role for *Bax* in lymphoma development in Lck-Bax transgenic mice.

Bcl-2 antagonizes tumor formation in Lck-Bax38/1 mice

Despite their antagonist function related to the susceptibility of cells to apoptosis, *Bax* and *Bcl-2* are both able to promote thymic lymphoma development. To examine whether these genes would cooperate in thymic lymphoma development or would be antagonistic, the rate of lymphoma formation in mice positive for both Lck-Bax and Lck-Bcl-2 was determined. The presence of the Lck-Bcl-2 transgene significantly delayed tumor development in Lck-Bax38/1 double transgenic mice (Figure 6). This was true for both *p53* -/- and *p53* +/- animals. This data demonstrate that lymphoma development from overexpression of *Bax* is delayed by coexpression of *Bcl-2*. This is most remarkable in the *p53* +/- mice where the control *p53* +/- mice get tumors relatively late³³ in comparison with Lck-Bax38/1 double transgenic (Figure 2). In these animals *Bcl-2* dramatically delays tumor formation with the 50% survival time going from ~6 months to >1 year (duration of the study) in the mice coexpressing *Bcl-2*.

Discussion

The demonstration that human *Bax* can be a transcriptional target of *p53* led to a model where *Bax* is a downstream effector of *p53*-dependent apoptosis.¹³ As *p53* is the most frequently mutated gene in human tumors, this makes *Bax* a strong candidate for a tumor suppressor. A number of studies in mice and humans support this hypothesis and suggest that *Bax* expression inhibits cancer formation. In animal studies, *Bax*-deficiency potentiates tumors development in SV40 Tag mice^{15,16} and facilitates E1A³⁴ transformation of fibroblasts. Studies of human malignancies have also implicated *BAX* as an important tumor suppressor. For example, in colorectal cancer,¹⁹ gastric carcinoma,³⁵ and acute lymphoblastic leukemia³⁶ frameshift mutations in *BAX* have been reported.

Figure 3 Aneuploidy in Lck-Bax-*p53*^{-/-} mice. **(a)** Shown are examples of DNA profiles from *p53* deficient mice without (left) and with (right) the Lck-Bax transgene. Multiple abnormal peaks are readily observed in the Lck-Bax transgenic mice. The age of each animal and the total number of thymocytes are indicated in the upper right-hand corner of each histogram **(b)** Using the assay illustrated in **(a)**, *p53*-deficient mice of the indicated Lck-Bax genotype were determined to be either diploid (open diamonds) or aneuploid (filled triangles) and this was plotted versus the total number of thymocytes. Animals with obviously enlarged thymi were excluded from this data and that described in Table 2. **(c)** Cytogenetic analysis of freshly isolated thymocytes from a 8.9-week-old Lck-Bax38/1-*p53*-deficient animal (panel A, lower right) is indicated. Both normal and abnormal populations were observed

Table 2 Increased aneuploidy in Lck-Bax mice

Lck-Bax	#Aneuploid	# Normal	Percentage
Neg	1	8	11.1%
Lck-Bax38, 1 or Both	9	7	56.25%

P=0.0405 by two-sided Fisher exact test

In colon cancer cell lines homozygous deletion of *Bax* promotes resistance of cell to chemotherapeutic agents and nonsteroidal anti-inflammatory drugs.¹⁷ Mutations in *Bax* have been described in a number of human hematopoietic

tumor cell lines³⁶ as well as directly from gastrointestinal cancer.³⁷ Retrospective studies of human malignancies have examined the relation between BAX expression by immuno-histochemistry or immunoblotting and clinical outcome. As expected if *Bax* is a tumor suppressor, reduced expression of BAX is associated with a worse clinical outcome in ovarian cancer,³⁸ metastatic breast adenocarcinoma,³⁹ and squamous cell carcinoma.⁴⁰ Analogously, increased expression of BAX has been associated with a better prognosis in squamous cell carcinoma⁴¹ and acute myeloid leukemia.⁴²

In contrast, other studies have found a paradoxical effect of *Bax* on tumor formation. For example, increased *Bax* levels were associated with a higher rate of relapse in childhood

Figure 4 Bax-induced aneuploidy in mice with functional *p53*. DNA content analysis on thymocytes was performed on an 8-week-old Lck-Bax38/1-*p53* +/- (a) and a 31-week-old Lck-Bax38/1-*p53* +/+ (b). The total number of thymocytes isolated is indicated. For (a), when gated the small aneuploid peak represented 7.8% of the events while the diploid peak represent 76.9%. The data indicate an aneuploid population in the animal that totaled fewer than 400 000 cells

acute lymphocytic leukemia.³⁰ In non-Hodkins lymphoma, increased *Bax* levels were associated with decreased patient survival.³¹ In mice, *Bax* deficiency was unable to accelerate spontaneous tumor development or tumor development in *p53* -/- mice, while overexpression of *Bax* accelerated the development of thymic lymphomas in *p53* -/- mice.²⁹ As discussed in the introduction, *Bcl-2* also has been found to have paradoxical effects on oncogenesis.^{23–28} Taken together, these studies suggest that the effects of *Bcl-2* family members on *de novo* tumor formation, susceptibility to treatment and progression are complex and a simple model in which inhibition of apoptosis (by increased *Bcl-2* or decreased *Bax*) accelerates tumor development or progression may not always apply (Figure 7a).

Several possibilities may explain these paradoxical findings regarding *Bax* and *Bcl-2* on tumor progression. In our initial studies, we found that *Bax* expression increased proliferation of thymocytes²⁹ and others have reported that *Bax* increases proliferation of T cells following Con A stimulation.⁴³ These observations are complemented by a number of studies that demonstrate that *Bcl-2* inhibits proliferation of T cells,^{44–46} fibroblasts⁴⁵ and IL-3-dependent hematopoietic cell lines.⁴⁷ Although cell cycle regulation is a tantalizing prospect for mediating the oncogenic function of *Bax* and the antioncogenic function of *Bcl-2*, the results reported here, that chromosomal instability is an early consequence of *Bax*

expression, provides an alternative and perhaps more likely explanation for the paradoxical acceleration of lymphoma development in mice overexpressing *Bax*.

As carcinogenesis is a multistep process that requires multiple mutations, recent investigators proposed that cells must acquire genomic instability as an enabling characteristic of tumor cells.³² Genetic instability occurs at many levels including chromosome translocations, subtle sequence instability (sometimes termed microsatellite), gene amplification or chromosomal instability (CIN).⁴⁸ A number of genes involved in colon cancer are important in DNA mismatch repair and result in microsatellite instability interest.⁴⁸ Similarly, chromosomal translocations are frequently associated with lymphoma and leukemia. However, chromosomal instability or CIN is the most common type of genetic instability and is observed in nearly all types of tumors.⁴⁸ One could argue that CIN is a result of and not a cause of transformation. However, many colon cancers from patients with defects in DNA repair (microsatellite instability) frequently do not have CIN.⁴⁹ Consistent with CIN being causative in our model, aneuploidy is a frequent and very early manifestation of high expression of *Bax*.

Despite the nearly ubiquitous presence of CIN, the pathways leading to CIN during oncogenesis are poorly understood. *p53*-deficient cells frequently demonstrate CIN, however, it does not appear that *p53* deficiency is sufficient

Figure 5 Thymic tumors in Lck-Bax transgenic mice maintain Bax expression and demonstrate progressive aneuploidy. (a) Lysates were prepared from thymic tumors (T) or normal thymus N and analyzed by Western blot for Bax expression (top) or actin (bottom). Samples were prepared from Lck-Bcl-2 (+) tumors and multiple tumors from Lck-Bax transgenic mice (lines indicated by either 38 or 38/1). (b) DNA content analysis from two of the tumor samples shown in (a). Shaded histograms represent PI staining from control (diploid) animals performed in the same experiment. The bold line represents the tumor samples as indicated

Figure 6 Bax and Bcl-2 are antagonistic in tumor development. All mice expressed both copies of Lck-Bax (Bax38/1) and were either *p53* $-/-$ (left) or *p53* $+/-$ (right). Tumor development with (●) and without (○) expression of Lck-Bcl-2 is shown. The animals that survived for >50 weeks were censored and are indicated by open diamonds. The indicated *P*-value is based on the Mantel-Cox logrank test (Statview)

for CIN as a number of cell lines are *p53* deficient and do not display CIN.⁴⁹ Furthermore, aneuploidy and CIN appear earlier in carcinogenesis than *p53* mutations leading to the

suggestion that *p53* exacerbates CIN but is neither necessary nor sufficient for it.⁴⁸ Although Bax-induced lymphoma development is clearly accelerated by loss of *p53*, the present data demonstrate that lymphoma development and CIN occur in animals with intact *p53*. Of note, tumor development in Lck-Bax38/1 was not accelerated in *p53* $+/-$ mice. This suggest that loss of *p53* is not rate limiting in Lck-Bax38/1 transgenic mice with functional *p53*.

Fanconi Anemia is a human inherited disease associated with chromosome instability that may be relevant to the results described here.⁵⁰ Fanconi Anemia is an autosomal recessive disease associated with skeletal birth defects, anemia, hypogonadism, reduced fertility and cancer (leukemia and squamous cell carcinoma). Fanconi anemia is a genetic heterogeneous disease with at least eight proposed complementation groups based on cell fusion studies. Subsequently, seven genes (FANCA, C, D1, D2, E, F and G) have been identified and associated with Fanconi Anemia.⁵¹ Although unrelated at the sequence level, these gene products form a multiprotein nuclear complex whose function remains largely

Figure 7 Models for relation between apoptosis and chromosomal instability. Current data support a model (a) in which chromosome instability (CIN) leads to increased apoptosis. In this model, inhibition of apoptosis by increase in Bcl-2 or decrease in Bax accelerate tumor development. The data described here support an alternative model (b) in which increased apoptosis (perhaps by changes in the Bcl-2 family) may directly result in CIN and accelerate oncogenesis. It should be noted that these models are not mutually exclusive but instead suggest alternative means to malignant transformation

unknown.⁵¹ Mutations in Fanconi A and C are most common, and animal models now exist for these diseases. FANCC has been perhaps the most widely studied and several of these studies are worth noting relevant to our work. Like *Bax*, FANCC is localized predominantly to the cytoplasm and this localization may be crucial for its function.^{52,53} Furthermore, similar to overexpression of *Bax*, loss of function of FANCC leads to increased apoptosis in hematopoietic cells^{54–56} and germ cells. Therefore, both Fanconi Anemia and our Lck-*Bax* model are associated with increased apoptosis and CIN. One proposed common mechanism by which these pathways may lead to CIN may be via regulation of reactive oxygen species. Of note, both FANCC⁵⁷ and *Bax*⁵⁸ may regulate cell death through glutathione S transferase. Although the significance of both these studies relative to the apoptotic function of the respective proteins remain controversial, the data demonstrate similarities between the two pathways, which may provide mechanistic insight into the pro-oncogenic activity of *Bax*.

Other studies have reported a connection between cell death and chromosome instability. Chinese hamster ovary cells that are unstable following radiation treatment show an increased susceptibility to apoptosis and increased oxidative stress.^{59,60} However, it was not clear in these studies whether increased susceptibility to apoptosis results from or perhaps precedes chromosome instability. Although alternative explanations exist, we favor a model in which *Bax* expression leads to CIN (directly or indirectly) while *p53* functions to delete or inactivate cells that have rearranged or damaged DNA (Figure 7). Inhibition of *Bax*-induced lymphoma development by *Bcl-2* supports a model in which increased sensitivity to apoptosis may lead to chromosome instability in developing T cells. These results support other studies that have found a correlation between chromosome instability and apoptosis and suggest that increased sensitivity to apoptosis may precede and in some cases cause chromosome instability (Figure 7b).

Consistent with this model, other studies have found that thymic lymphomas develop in a setting of increased apoptosis. For example, Rag-1 and Rag-2 deficiency accelerated thymic lymphoma development in *p53* $-/-$ mice.⁶¹ Rag-1/2 are essential for rearrangement of the T-cell receptor and are

therefore necessary for T-cell development.⁶² Since thymocytes are unable to mature, the rate of apoptosis is increased because of what is sometimes referred to as death by neglect. Deficiency in an orphan steroid receptor results in increased apoptosis and T-cell lymphomas in mice.⁶³ In humans, immunosuppressive therapy and corticosteroid may increase the risk of developing non-Hodgkins lymphoma.⁶⁴ Taken together, these results suggest that genes (Bcl-2 family members) or factors that regulate apoptosis may regulate tumor development by controlling chromosome instability. They support a model in which increased sensitivity to apoptosis may precede and in some cases cause chromosome instability (Figure 7b).

Materials and Methods

Materials and mice

Lck-*Bcl-2* and Lck-*Bax* transgenic mice were previously described and are genotyped by polymerase chain reaction (PCR) as previously described.^{29,65} *P53*-deficient mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) were genotyped by PCR as previously described.^{33,66} Lck-*Bax38* and Lck-*Bax1* transgenic mice could be distinguished from one another because of the presence of a longer 3' untranslated domain in the cDNA for Lck-*Bax1*. This results in a larger PCR product (~150 bp) for Lck-*Bax1* relative to Lck-*Bax38*.

Tumor development studies

All mice were maintained in the animal facility at the University of Iowa under an approved protocol (ACURF #9807226). Mice of the appropriate genotypes were mated to obtain animals for the tumor development studies. Upon entry into the tumor development study, animals were examined weekly for signs of illness or malignancy. Sick animals were monitored more frequently and euthanized when necessary to prevent unnecessary suffering. Control animals were monitored for up to 1 year and then killed for necropsy. As these animals did not show overt signs of disease, they were all censored from the analysis. When possible, necropsies were performed on dead animals to determine if the animals had gross evidence of tumors. Tumors were then confirmed by fixation with formalin and histological examination after H&E staining. All mice were maintained in the animal facility at the University of Iowa (Iowa City, IA, USA). Statistical analysis was performed with the StatView Program (SAS Institute Inc.) using Kaplan–Meier cumulative survival and the logrank (Mantel–Cox) test to determine the likelihood that differences in survival were significant between each of the indicated groups.

Cell preparation and analysis

Single-cell suspensions were prepared from the thymus by dispersing the organs between two glass slides in isotonic saline. In some experiments, red blood cells were removed by a 5 min incubation in hypotonic lysis buffer (0.83% NH_4Cl , 10 mM Tris/pH 7.2).⁴⁴ Viable cell counts were determined using a hemocytometer and trypan blue exclusion. Cells were cultured in RPMI supplemented with 10% FCS, Pen–Strep, glutamine and 2-mercaptoethanol (100 μM). Cell cycle analysis was performed by analyzing propidium iodide-stained nuclei on a flow cytometer equipped for doublet discrimination (FACScan or FACSCalibur from Becton Dickinson). Briefly, 1 million cells were pelleted and resuspended in 0.5 ml of Krishan reagent prior to analysis by flow cytometry.⁶⁷ Doublet

events were gated out based on FL-2 area versus FL-2 width. Cellquest software (Becton Dickinson) was used for both acquisition and analysis.

Cytogenetics

Cytogenetics was performed with the assistance of the cytogenetics laboratory at the University of Iowa. Briefly, thymocytes were isolated as described above and cultured briefly in colcemid (Gibco-BRL Cat#15210-040). The thymus organ was extracted from 8.9-week-old mice. The organ was minced and the thymocytes were treated with colcemid (Gibco-BRL Cat#15210-040) at 1 µg/ml for 30 min at 37°C. Following colcemid treatment, thymocytes were hypotonically swollen in 75 mM KCl and fixed in 3:1 acetic acid:methanol. Metaphase spreads were obtained by dropping fixed cells onto wet slides stored at 4°C. The slides were stained with Giemsa prior to photography.

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