Functional antagonism between pro-apoptotic BIM and anti-apoptotic BCL-XL in MYC-induced lymphomagenesis

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Abstract:

Genomic analyses revealed that many cancers have acquired abnormalities in their expression of pro- or anti-apoptotic members of the BCL-2 protein family. It is, however, unknown whether changes in pro- or anti-apoptotic BCL-2 family members have similar impact on tumorigenesis or whether changes in one subgroup have disproportionate impact. We compared the impact of concomitant loss of anti-apoptotic *Bclx* and pro-apoptotic *Bim* on MYC-induced lymphomagenesis. Whereas only loss of both *Bclx* alleles markedly forestalled tumorigenesis, loss of a single *Bim* allele overcame this blockade. Conversely, loss of even a single *Bim* allele sufficed to substantially accelerate lymphomagenesis, and only loss of both but not loss of a single allele of *Bclx* could attenuate this acceleration. The evidence that modest (2-fold) mono-allelic changes in expression of at least some BH3-only proteins can profoundly impact tumorigenesis suggests that such aberrations, imposed by epigenetic or genetic changes, may expedite tumorigenesis more effectively than elevated expression of pro-survival BCL-2 family members. These findings further our understanding of the mechanisms of lymphomagenesis and possibly also cancer therapy.

Introduction:

Defects in apoptosis promote tumorigenesis, particularly together with oncogenic lesions that drive excess cell proliferation [1]. Apoptosis is controlled by three distinct subgroups of the BCL-2 protein family: the pro-survival proteins (e.g. BCL-2, BCL-XL, MCL-1) are essential for cell survival; the BH3-only proteins (e.g. BIM, PUMA) are crucial for apoptosis initiation; finally, BAX/BAK are required for apoptosis execution [2]. Loss of anti-apoptotic BCL-XL incites failure of embryonic erythropoiesis and excess neuronal attrition [3], whereas deficiency of the pro-apoptotic BH3-only protein BIM causes excess lymphocyte accumulation with predisposition to lymphoid malignancy [4] and autoimmune disease [5]. Remarkably, BIM deficiency prevents the erythropoietic defects caused by BCL-XL loss [6]. This functional antagonism reflects their high affinity physical interaction [2, 7].

Many human cancers display abnormalities in the expression of anti-apoptotic or pro-apoptotic BCL-2 family members. *BCL-2* is over-expressed in follicular centre B cell lymphoma due to its chromosomal translocation into the *IGH* locus [8] and DNA regions containing the *BCL-X* or *MCL-1* genes are amplified in diverse human cancers [9]. Conversely, *BIM* is lost in 17% of mantle cell lymphoma [10] and *BIM* or *PUMA* are frequently silenced epigenetically in diverse tumors [11, 12]. Accordingly, overexpression of anti-apoptotic BCL-2 [1] or BCL-XL [13] or loss of pro-apoptotic BIM [4] or PUMA [14] greatly accelerates MYC-induced lymphomagenesis in transgenic mice.

Despite the wealth of genomic information on abnormalities in the expression of pro- or anti-apoptotic BCL-2 family proteins in cancer, it is not clear whether such abnormalities exert equal impact on tumorigenesis or whether one subgroup of the BCL-2 family exerts disproportionate effects. We therefore examined the functional interplay between BCL-XL and BIM during pre-B/B lymphoma development in *Eμ-Myc* transgenic mice [15].

Results and Discussion:

Impact of loss of a single allele of either *Bclx* or *Bim* on MYC-driven lymphoma development

Consistent with published data [16], loss of one *Bclx* allele slightly ($p = 0.049$) delayed lymphoma onset (median survival: *Eμ-Myc;Bclx*$^{+/-}$ 184 days vs *Eμ-Myc* 145 days; Figure 1A). Conversely, loss of one *Bim* allele markedly accelerated lymphomagenesis (median survival: *Eμ-Myc;Bim*$^{+/-}$ 78 days vs *Eμ-Myc* 145 days, $p < 0.001$; Figure 1A), as reported [4]. Remarkably, in the context of *Bclx* heterozygosity, loss of a single *Bim* allele accelerated the lymphomagenesis to a rate indistinguishable from that of *Eμ-Myc;Bim*$^{+/-}$ mice (median survival: *Eμ-Myc;Bclx*$^{+/-}$*;Bim*$^{+/-}$ 75 days vs *Eμ-Myc;Bim*$^{+/-}$ 78 days, $p = 0.0736$), significantly ($p < 0.001$) faster even than the 145 days in control *Eμ-Myc* mice (Figure 1A). At autopsy, lymphoma-bearing mice of all genotypes had comparable blood leukocyte numbers and organ weights, except for a minor increase in spleen weight in the *Eμ-Myc;Bim*$^{+/-}$ mice, relative to the *Eμ-Myc* mice (Figure 1B,C).

These results show that loss of one *Bim* allele was sufficient to not only overcome the delay in lymphomagenesis imposed by loss of one *Bclx* allele, but was able to markedly accelerate tumor development, even beyond the rate seen in control *Eμ-Myc* mice.

Loss of a single *Bim* allele is sufficient to overcome the delay in lymphoma development caused by the complete absence of BCL-XL

Given that profound inhibition of MYC-induced lymphomagenesis requires the complete absence of BCL-XL [16], we investigated the antagonism between BIM and BCL-XL in this setting (Figure 2). For this, lethally irradiated C57BL/6-Ly5.1 congenic mice were transplanted with a mixture of fetal liver-derived stem cells from E12.5 embryos of C57BL/6-Ly5.2 *Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{-/-}$ (or control) genotypes, to replenish lymphopoiesis, plus stem cells from *Rag1*$^{-/-}$ (C57BL/6-Ly5.2) mice, to restore erythropoiesis (and myelopoiesis) (Figure 2A and Figure S1A). By the analysis endpoint (1 year), 47% of (control) mice reconstituted with an *Eμ-Myc* lymphoid system had developed lymphoma. Loss of BCL-XL almost abrogated lymphomagenesis, with 85% of *Eμ-Myc*;*Bclx*$^{-/-}$ reconstituted mice remaining tumor-free (Figure 2B). We attribute the somewhat higher lymphoma incidence (15%) here compared to our previous study [16] to the more efficient hematopoietic reconstitution. Loss of BIM greatly accelerated lymphomagenesis: the median lymphoma-free survival period for mice with an *Eμ-Myc;Bim*$^{-/-}$ lymphoid system was 76 days and for the *Eμ-Myc;Bim*$^{+/-}$ reconstituted animals it was 126 days (Figure 2C and Figure S1B).

We assessed the importance of the BIM/BCL-XL antagonism on MYC-induced lymphomagenesis from two perspectives. The first is the ability of reduced BIM levels to overcome the reduction in incidence and the delay in onset of lymphomagenesis caused by loss of BCL-XL (Figure 2B). Compared to *Eμ-Myc;Bclx*$^{-/-}$ reconstituted mice (15% incidence), those whose lymphoid system also lacked one or both *Bim* alleles developed lymphomas much faster and with substantially higher incidence (*Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{+/-}$; 75% incidence, median survival 214 days: $p = 0.0074$; *Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{-/-}$, 80% incidence, median survival 135 days: $p = 0.0006$).

The second perspective is the ability of reduced BCL-XL levels to countermand the acceleration in lymphomagenesis provoked by the loss of BIM (Figure 2C). Remarkably, loss of a single *Bclx* allele was unable to significantly curb the acceleration in lymphomagenesis driven by loss of one *Bim* allele (median survival *Eμ-Myc;Bclx*$^{+/-}$*;Bim*$^{+/-}$ 131 days vs *Eμ-Myc;Bim*$^{+/-}$ 121 days; p = 0.1162). A significant delay in lymphoma development could only be achieved by loss of both *Bclx* alleles (*Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{+/-}$ median survival 214 days, p < 0.0001). The tumor burden at sacrifice tended to be comparable for mice of all genotypes albeit with somewhat greater spleen weights in the mice deficient for BCL-XL. This was probably due to their enhanced survival and hence older age and larger overall body size at analysis (Figure 2D).

Antagonism of pro-apoptotic BIM is critical for lymphoma development in *Eμ-Myc* mice

Collectively, our results demonstrate that BIM exerts a proportionally greater influence on lymphoma development compared to BCL-XL. The paradigm that relatively subtle reductions in the levels of (certain) BH3-only family members allows cells to effectively evade apoptosis while undergoing neoplastic transformation, thereby promoting tumorigenesis, may be applicable to other tumor contexts, but the antagonistic pairs of BCL-2 family members are likely to vary between different cancers. For example, loss of neither BCL-XL nor BIM affected γ-irradiation-induced thymic lymphoma development (Figure 3 and Figure S2). In that model, antagonism between PUMA and MCL-1 might instead control lymphomagenesis, since both of these BCL-2 family members regulate, in a mutually antagonistic manner, the survival of bone marrow derived hematopoietic stem/progenitor cells [17-19], the cells of origin for this malignancy [20].

These results re-enforce the concept that BIM-mediated apoptosis constitutes a potent tumor suppressive mechanism, at least within the context of lymphoma development (Figure 4) [4, 21]. Loss of BCL-XL increases the propensity of nascent pre-leukemic cells to undergo apoptosis [16]; thus, other pro-survival BCL-2 family members must allow rare *Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{+/-}$ nascent leukemic clones to resist

BIM-mediated apoptosis and eventually become malignant lymphoma cells. Our finding that a modest reduction in BIM expression can substantially increase the incidence and onset of lymphoma development, even when pro-survival BCL-2 family protein expression is compromised (here due to loss of BCL-XL) indicates that relatively subtle changes in the expression of BH3-only proteins are likely to be clinically relevant. Techniques to reliably detect such subtle changes may therefore have utility for cancer diagnosis and may even provide information on therapeutic strategies.

Implications for cancer therapy

The observation that relatively subtle reductions in BH3-only proteins (here a reduction in BIM levels) can have substantial impact on tumor development is likely to also be relevant to cancer therapy. Killing of tumor cells by conventional (genotoxic) cancer drugs requires PUMA, NOXA and BIM [22]. BIM is also critical for the therapeutic efficacy of "designer" drugs that inhibit oncogenic kinases [23] and even the BH3 mimetics ABT-737 and ABT-263, which target anti-apoptotic BCL-2, BCL-XL and BCL-W [24]. Interestingly, a 4-week-treatment of pre-leukemic *Eμ-Myc* mice with ABT-737 potently inhibited lymphomagenesis [25], presumably through a BIM-dependent process following its release from antagonism by BCL-XL (BCL-2 and BCL-W do not play a role in this context [26, 27]). Thus, our results indicate that therapeutic modalities that reactivate BIM in cancer cells in which its expression is repressed (e.g. due to epigenetic modifications) represent a promising strategy for the treatment of MYC-driven lymphomas and perhaps MYC-driven cancers more generally. The impact of such treatments is expected to be boosted by selective BH3 mimetics [23].

Conflict of interest: The authors are employed by The Walter and Eliza Hall Institute. The Walter and Eliza Hall Institute receives milestone payments from Genentech and AbbVie for the development of ABT-199 for cancer therapy.

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Figure Legends:

Figure 1. Loss of one allele of *Bim* not only overcomes the delay in MYC-induced lymphoma development imposed by loss of one allele of *Bclx* but accelerates the

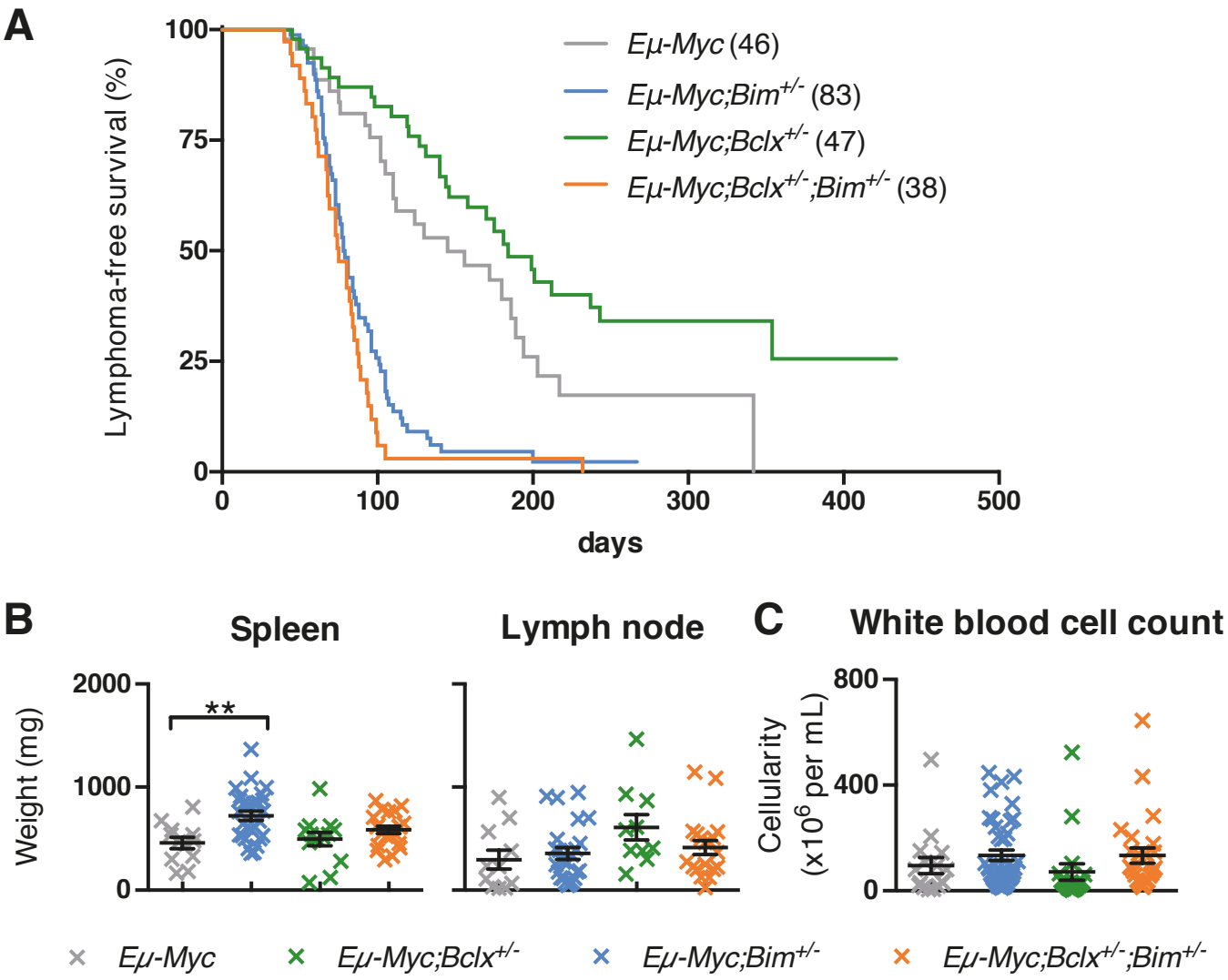
rate beyond that of control *Eμ-Myc* mice. (**A**) Kaplan-Meier survival curves showing the rates of lymphoma development in *Eμ-Myc* (control), *Eμ-Myc;Bim*$^{+/-}$, *Eμ-Myc;Bclx*$^{+/-}$ and *Eμ-Myc;Bclx*$^{+/-}$*;Bim*$^{+/-}$ mice. (**B**) Spleen, and lymph node weights and (**C**) peripheral white blood cell counts were compared between lymphoma-bearing *Eμ-Myc*, *Eμ-Myc;Bim*$^{+/-}$, *Eμ-Myc;Bclx*$^{+/-}$, and *Eμ-Myc;Bclx*$^{+/-}$*;Bim*$^{+/-}$ mice. Data are presented as mean+/-SEM. ** denotes significant differences where $p < 0.01$ (one-way ANOVA, Bonferroni correction for multiple comparisons). Experiments with mice were conducted according to the guidelines and with approval from the Walter and Eliza Hall Institute for Medical Research Animal Ethics Committee. *Eμ-Myc* transgenic (generated on a mixed C57BL/6xSJL background by oocyte injection) [15], *Bclx*$^{-/-}$ [3] and *Bim*$^{-/-}$ [5] mice (the latter two generated on a mixed C57BL/6x129SV background using 129SV derived ES cells) have been previously described. All strains were backcrossed to C57BL/6 mice for >20 generation prior to the current study. Compound mutant mice were generated by inter-crossing the relevant transgenic and single knockout strains.

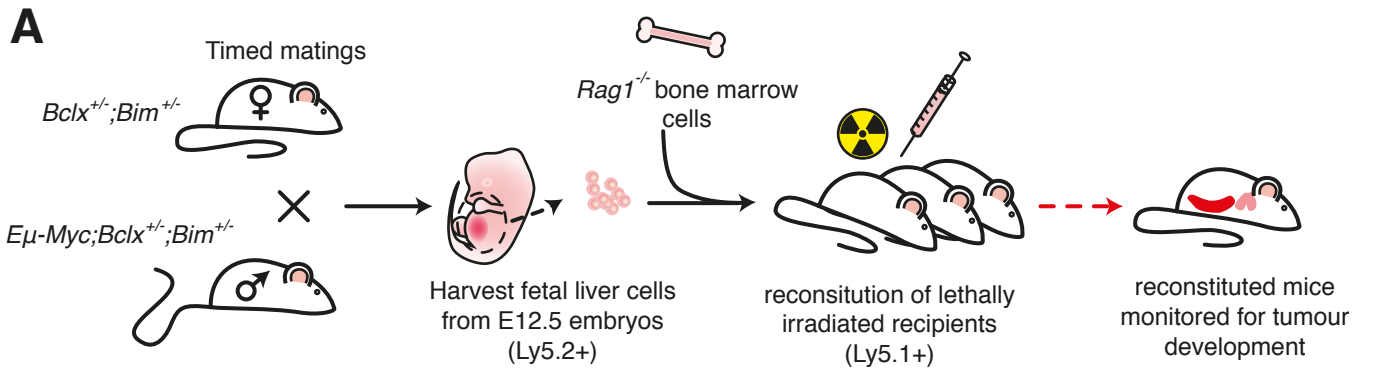
Figure 2. Antagonism between BIM and BCL-XL controls the rate of MYC-induced lymphomagenesis. (**A**) Hematopoietic reconstitution strategy. *Eμ-Myc;Bclx*$^{-/-}$, *Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{-/-}$ and relevant control embryos were generated through timed matings of *Eμ-Myc;Bclx*$^{+/-}$*;Bim*$^{+/-}$ males with *Bclx*$^{+/-}$*;Bim*$^{+/-}$ females. Fetal livers were harvested from E12.5 embryos and hematopoietic reconstitution performed as previously described [16]. Briefly, each fetal liver cell suspension was supplemented with 3 x 10^5 *Rag1*$^{-/-}$ bone marrow cells and the mixture injected intravenously (i.v.) into 3 lethally irradiated (2 x 5.5 Gy, at 3 h interval) C57BL/6-Ly5.1 recipient mice. Recipient mice were maintained on neomycin sulphate supplemented drinking water for 28 days post-irradiation to prevent infection. Mice were monitored thrice weekly for signs of lymphoma development (lymphadenopathy, splenomegaly, respiratory difficulties) until 1 year post-transplantation. (**B-C**) Kaplan-Meier lymphoma-free survival curves of mice reconstituted with fetal liver derived stem/progenitor cells of the genotypes: *Eμ-Myc* (control), *Eμ-Myc;Bim*$^{+/-}$, *Eμ-Myc;Bclx*$^{+/-}$, *Eμ-Myc;Bclx*$^{+/-}$*;Bim*$^{+/-}$, *Eμ-Myc;Bclx*$^{-/-}$, *Eμ-Myc*;*Bclx*$^{-/-}$*;Bim*$^{+/-}$ and *Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{-/-}$. Animal survival data are presented

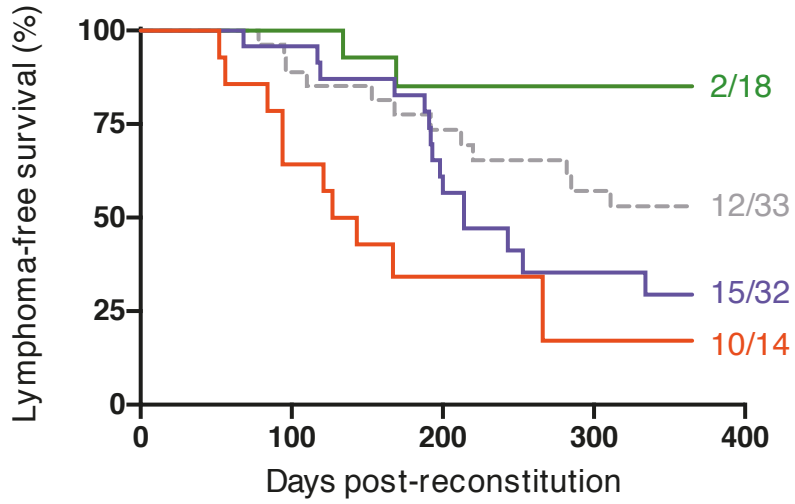
from two perspectives. (**B**) Loss of either one or both alleles of *Bim* not only overcame the delay in lymphoma onset caused by the absence of *Bclx* but accelerated lymphoma onset even beyond that of control *Eμ-Myc* mice. (**C**) Loss of even a single *Bim* allele substantially accelerated lymphoma development compared to *Eμ-Myc* reconstituted mice. This acceleration could only be reverted by loss of both *Bclx* alleles, whereas loss of a single *Bclx* allele was unable to antagonize this acceleration in lymphoma development. Note the data shown in (**B**) and (**C**) were generated in parallel, the *Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{+/-}$ and *Eμ-Myc* survival curves are shown on both panels to aid comparison to the other genotypes. (**D**) Lymphoma burden was assessed in *Eμ-Myc*, *Eμ-Myc;Bim*$^{+/-}$, *Eμ-Myc;Bclx*$^{+/-}$, *Eμ-Myc;Bclx*$^{+/-}$*;Bim*$^{+/-}$, *Eμ-Myc;Bclx*$^{-/-}$, *Eμ-Myc*;*Bclx*$^{-/-}$*;Bim*$^{+/-}$ and *Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{-/-}$ reconstituted mice when sick. In each case the spleen and lymph node weights, and peripheral white blood cell count were recorded. Data are presented as mean+/-SEM. * and ** denote significant differences where $p < 0.05$ and $p < 0.01$, respectively (one-way ANOVA, Bonferroni correction for multiple comparisons).

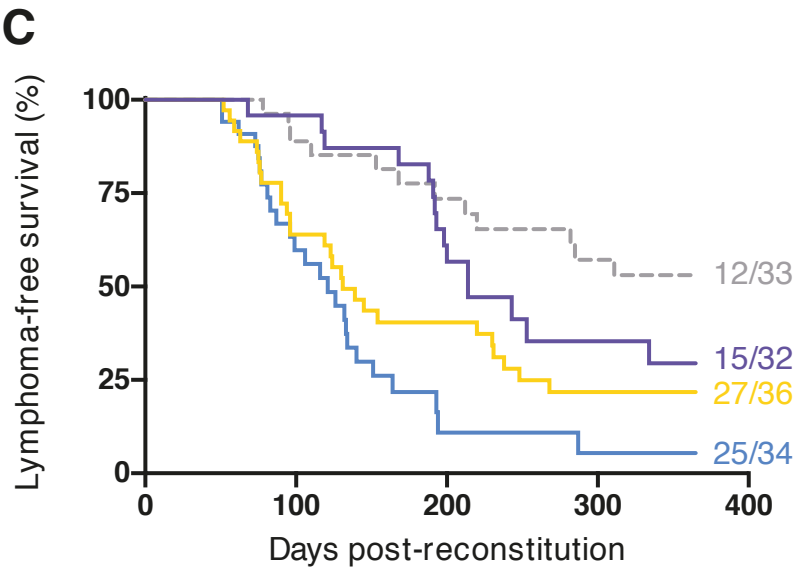
Figure 3. BIM and BCL-XL are not critical determinants of γ-irradiation-induced thymic lymphoma development. Kaplan-Meier survival curves showing survival of wild-type, *Bim*$^{+/-}$, *Bclx*$^{+/-}$ and *Bclx*$^{+/-}$*;Bim*$^{+/-}$ littermates following 4 weekly doses of γ-irradiation (1.5 Gy) starting at 1 month of age to elicit thymic lymphoma development. Mice were monitored until 400 days of age to determine their rates of lymphoma incidence. No significant differences in lymphoma onset were observed [log-rank (Mantel-Cox) test].

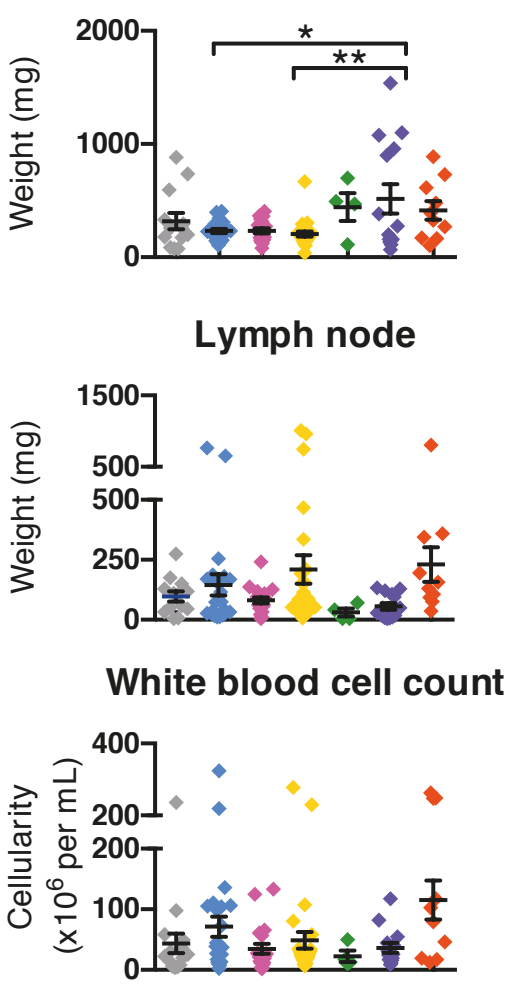
Figure 4. Model depicting the regulation of MYC-driven lymphoma development by the battle between BIM and BCL-XL. In the context of MYC-overexpression loss of BIM promotes lymphoma development by protecting nascent leukemic cells from apoptosis elicited by oncogenic stress (e.g. deregulated MYC expression), functioning as a rate-limiting tumor suppressive process. In cells that evade BIM-induced apoptosis MYC overexpression will promote excess growth and proliferation, thereby driving lymphoma progression.


A
Lymphoma-free survival (%)
100
75
50
25
0
0
100
200
300
400
500
days
Eμ-Myc (46)
Eμ-Myc;Bim+/- (83)
Eμ-Myc;Bclx+/- (47)
Eμ-Myc;Bclx+/-;Bim+/- (38)
B
Spleen
Lymph node
Weight (mg)
2000
1000
0
**
C
White blood cell count
Cellularity (x10^6 per mL)
800
400
0
Eμ-Myc
Eμ-Myc;Bclx+/-
Eμ-Myc;Bim+/-
Eμ-Myc;Bclx+/-;Bim+/-



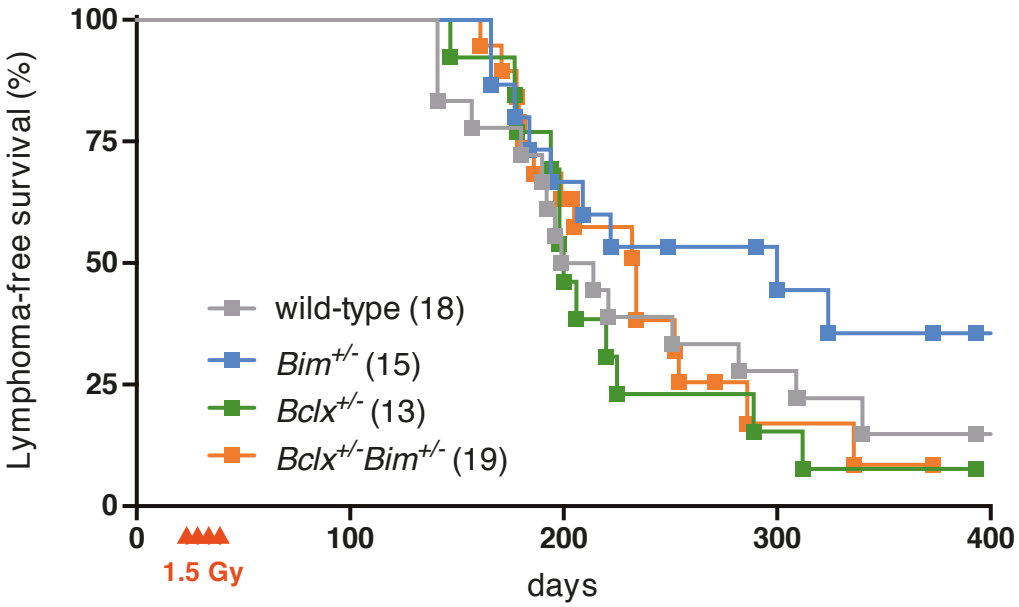


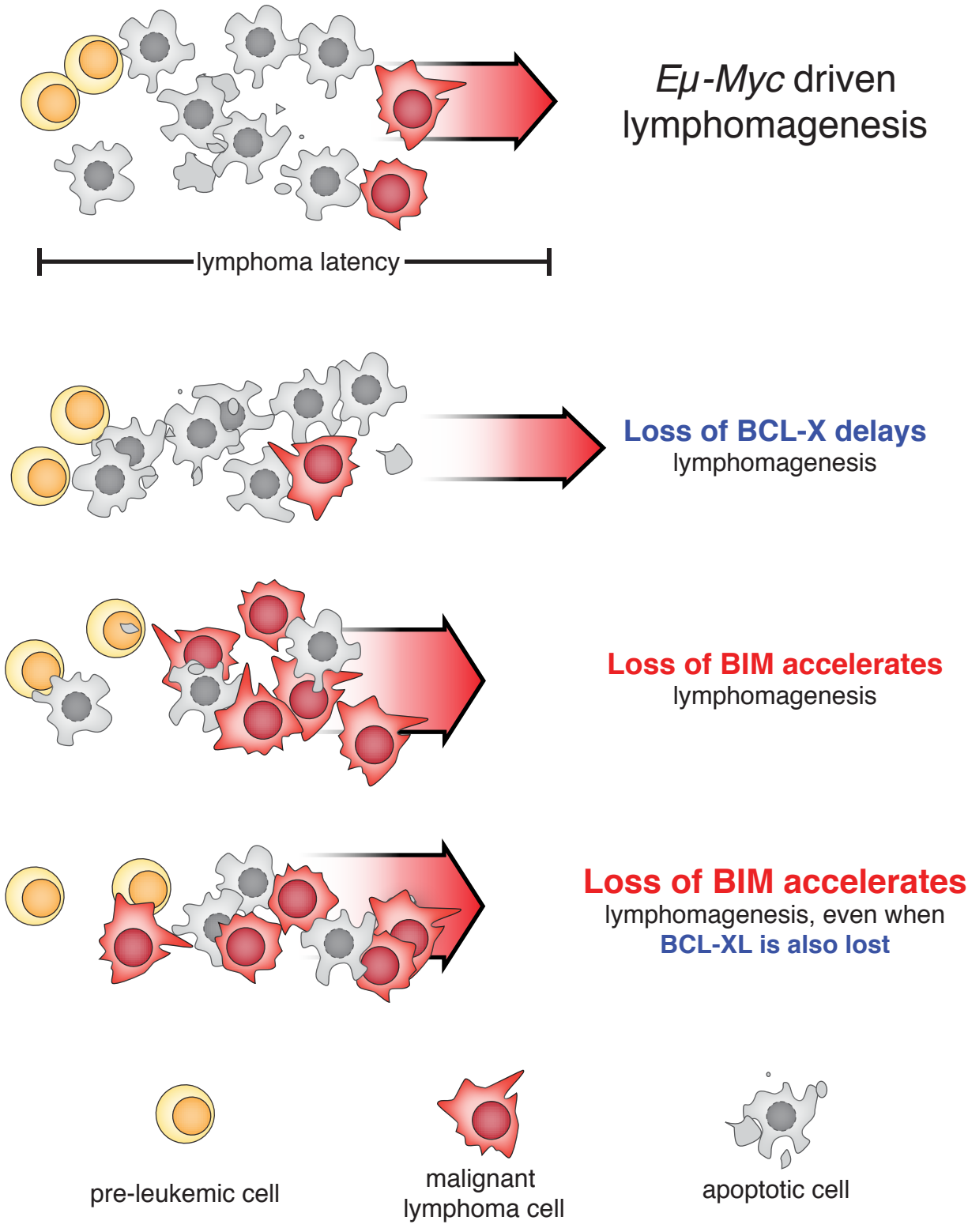




Eμ-Myc
Eμ-Myc;Bim+/-
Eμ-Myc;Bclx+/-
Eμ-Myc;Bclx+/-;Bim+/-

Eμ-Myc;Bclx-/-
Eμ-Myc;Bclx-/-;Bim+/-
Eμ-Myc;Bclx-/-;Bim-/-


Lymphoma-free survival (%)
100
75
50
25
0
0
100
200
300
400
days
1.5 Gy
wild-type (18)
Bim+/- (15)
Bclx+/- (13)
Bclx+/-Bim+/- (19)


Eμ-Myc driven lymphomagenesis
lymphoma latency
Loss of BCL-X delays lymphomagenesis
Loss of BIM accelerates lymphomagenesis
Loss of BIM accelerates lymphomagenesis, even when **BCL-XL is also lost**
pre-leukemic cell
malignant lymphoma cell
apoptotic cell

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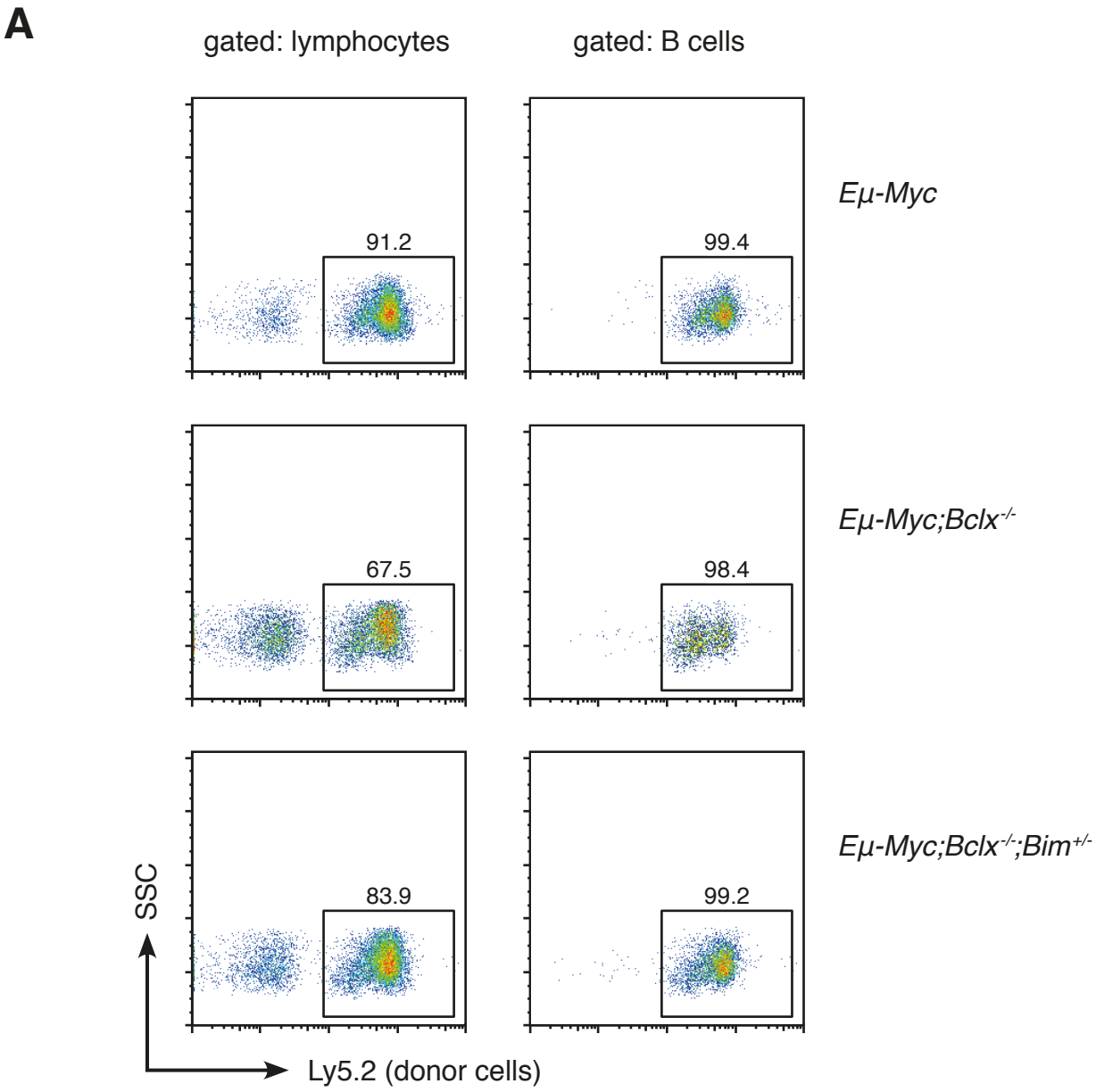
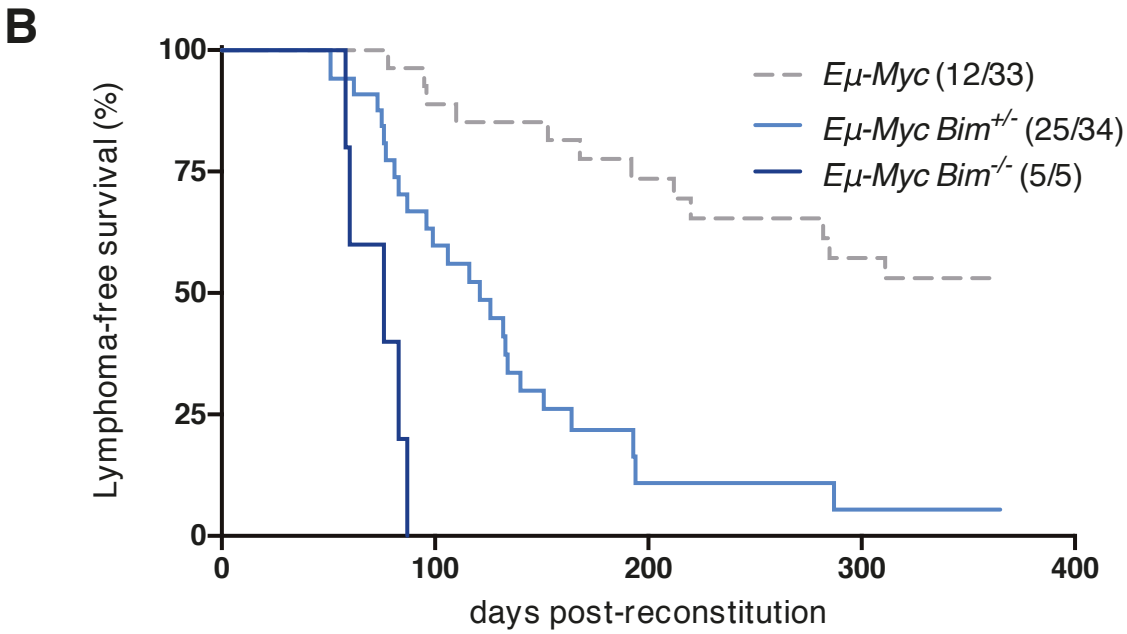
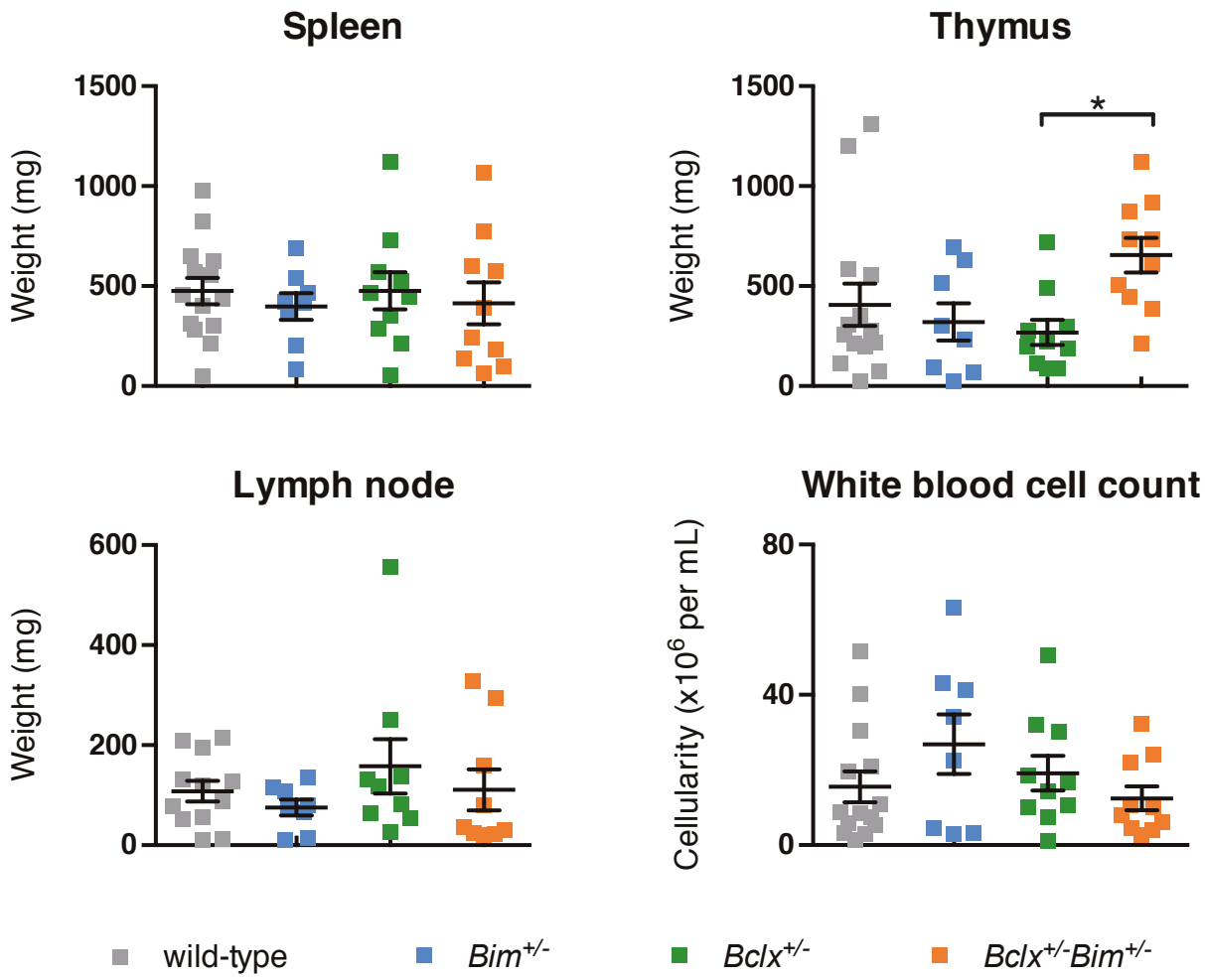
Supplementary Figure Legends

Figure S1. Reconstitution of the hematopoietic compartment of lethally irradiated wild-type mice by transplantation with *Eμ-Myc* fetal liver cells. (**A**) *Eμ-Myc;Bclx*$^{-/-}$ fetal liver derived stem/progenitor cells could efficiently repopulate the hematopoietic system of lethally γ-irradiated C57BL/6-Ly5.1 mice. Peripheral blood was harvested from lethally irradiated C57BL/6-Ly5.1 mice that had been co-reconstituted with stem/progenitor cells from fetal livers of *Eμ-Myc*, *Eμ-Myc;Bclx*$^{-/-}$ or *Eμ-Myc;Bclx*$^{-/-}$*;Bim*$^{+/-}$ E12.5 C57BL/6-Ly5.2 embryos and bone marrow cells from *Rag-1*$^{-/-}$ mice 8 weeks earlier and analyzed to determine the level of donor-derived leukocytes. Representative FACS plots are shown. (**B**) Loss of BIM accelerated *Eμ-Myc* induced lymphoma onset in hematopoietic reconstituted mice. Kaplan-Meier survival curves showing the effect of loss of BIM in mice reconstituted with an *Eμ-Myc* hematopoietic system. Loss of one or both alleles of *Bim* provoked a significant acceleration in MYC-driven lymphoma development (*Eμ-Myc;Bim*$^{+/-}$ 121 days median survival *vs Eμ-Myc* 53% survival at endpoint, $p < 0.0001$; *Eμ-Myc;Bim*$^{-/-}$ 76 days median survival vs *Eμ-Myc* 53% survival at endpoint, $p < 0.0001$).

Figure S2. Loss of a single allele of *Bim* and/or *Bclx* does not significantly alter lymphoma burden during γ-irradiation-induced thymic lymphoma development. Wild-type, *Bim*$^{+/-}$, *Bclx*$^{+/-}$ and *Bclx*$^{+/-}$*;Bim*$^{+/-}$ littermates were subjected to 4 weekly doses of γ-irradiation (1.5 Gy) starting at 1 month of age to elicit thymic lymphoma development. Lymphoma burden was assessed when mice fell sick. In each case the thymus, spleen and lymph node weights, and peripheral white blood cell count were recorded. Data are presented as mean+/-SEM. * denotes significant differences where $p < 0.05$ (one-way ANOVA, Bonferroni correction for multiple comparisons).


A
gated: lymphocytes
gated: B cells
91.2
99.4
Eμ-Myc
67.5
98.4
Eμ-Myc;Bclx-/-
83.9
99.2
Eμ-Myc;Bclx-/-;Bim+/-
SSC
Ly5.2 (donor cells)


B
Lymphoma-free survival (%)
100
75
50
25
0
0
100
200
300
400
days post-reconstitution
Eμ-Myc (12/33)
Eμ-Myc Bim+/- (25/34)
Eμ-Myc Bim-/- (5/5)


Spleen
Weight (mg)
1500
1000
500
0
Thymus
Weight (mg)
1500
1000
500
0
*
Lymph node
Weight (mg)
600
400
200
0
White blood cell count
Cellularity (x10^6 per mL)
80
40
0
wild-type
Bim+/-
Bclx+/-
Bclx+/-Bim+/-