Published in final edited form as:
Oncogene. 2012 July 12; 31(28): 3392–3396. doi:10.1038/onc.2011.508.

Bim must be able to engage all pro-survival Bcl-2 family members for efficient tumor suppression

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Abstract

Over-expression of the transcriptional regulator Myc is thought to be the cause or a contributing factor in the development of a large number of human lymphomas and certain other cancers. Apoptotic cell death constitutes a tumor suppressive mechanism, particularly in the context of Myc over-expression. Accordingly, lymphoma development in Eμ-*Myc* transgenic mice, which mimic the *Myc/IgH* chromosomal translocation that causes Burkitt Lymphoma, is accelerated by concomitant over-expression of anti-apoptotic Bcl-2 family members or loss of proapoptotic BH3 only proteins, such as Bim. Bim binds with high affinity to all prosurvival Bcl-2-like proteins and can also interact with Bax/Bak, but it remains unclear which of these interactions are critical for its tumor suppressive function.

We have previously generated knock-in mutant mice in which the BH3 region of Bim has been exchanged with that for Bad, Noxa or Puma so that it can only bind to select pro-survival Bcl-2-like proteins: Bim^{Bad} binding to Bcl-2, Bcl-x_L and Bcl-w but not Mcl-1 or A1; Bim^{Noxa} binding only to Mcl-1 and A1 and as a control, Bim^{Puma}, which can still bind all pro-survival Bcl-2-like proteins. We have now inter-crossed these Bim mutant mice with Eμ-*Myc* transgenic mice and found that both the Bim^{Bad} and the Bim^{Noxa} mutations but not the Bim^{Puma} mutation greatly accelerate Myc-induced lymphoma development and increase leukemic burden. These results demonstrate that for optimal tumor suppressive activity, Bim must be able to interact with all and not just select pro-survival Bcl-2 family members.

Keywords

Myc; Bim; tumor suppression; lymphoma; apoptosis

Introduction

The oncogene *Myc* is over-expressed in ~70% of human cancers and the *Myc/IgH* chromosomal translocation is the cause of Burkitt Lymphoma (Pelengaris *et al.*, 2002). Eμ-*Myc* transgenic mice over-express Myc under control of the immunoglobulin heavy chain gene enhancer (Eμ), mimicking the *Myc/IgH* chromosomal translocation found in human Burkitt lymphomas (Adams *et al.*, 1985). Myc over-expression causes abnormally increased proliferation of B lymphoid cells (Langdon *et al.*, 1986) and acquisition of additional oncogenic lesions precipitates malignant clonal pre-B or B-cell lymphomas with a median latency of ~100 days (on a C57BL/6 background) (Michalak *et al.*, 2009).

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Conflict of interest statement

The authors have no conflict of interest to disclose.

In addition to promoting abnormally increased cell proliferation, Myc over-expression also enhances apoptotic cell death under conditions of stress, such as limited growth factor supply (Pelengaris *et al.*, 2002; Strasser *et al.*, 1996). This apoptosis imposes a barrier against Myc-induced neoplastic transformation (Pelengaris *et al.*, 2002) and, accordingly, concomitant over-expression of pro-survival Bcl-2-like proteins (Strasser *et al.*, 1990) or loss of pro-apoptotic relatives, such as Bim (Egle *et al.*, 2004) or Puma (Garrison *et al.*, 2008; Hemann *et al.*, 2004; Michalak *et al.*, 2009), greatly accelerates lymphomagenesis in Eμ-*Myc* mice. Proteins of the Bcl-2 family, which comprises three subgroups with distinct functions, are major regulators of apoptosis (Youle and Strasser, 2008). The pro-survival members (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1) are essential for cell survival, the BH3-only proteins (e.g. Bim, Puma, Bad, Noxa) initiate apoptosis signaling and Bax/Bak are required for mitochondrial outer membrane permeabilization (MOMP) and activation of the caspase cascade that dismantles the cells (Strasser *et al.*, 2011). The molecular mechanisms for Bax/Bak activation are not fully resolved but appear to involve both direct activation by BH3-only proteins as well as indirect activation by BH3-only protein mediated blockade of the pro-survival Bcl-2 family members (Chipuk and Green, 2008; Merino *et al.*, 2009; Strasser *et al.*, 2011). BH3-only proteins bind with their BH3 region to a groove on the surface of pro-survival Bcl-2 proteins, but individual members of this subgroup differ substantially in their binding specificity. Bim and Puma interact with all pro-survival proteins and this accounts (at least in part) for their potent pro-apoptotic activity (Chen *et al.*, 2005; Kuwana *et al.*, 2005). Conversely, Bad and Noxa appear to be only weak killers (at least when over-expressed), and this has been attributed to their limited binding to Bcl-2, Bcl-x_L and Bcl-w or Mcl-1 and A1, respectively (Chen *et al.*, 2005; Kuwana *et al.*, 2005). The binding specificity of BH3-only proteins is determined by their BH3 region (Chen *et al.*, 2005; Kuwana *et al.*, 2005). Therefore the importance of Bim's ability to bind all pro-survival Bcl-2-like proteins could be investigated by generating Bim BH3 region replacement mutant knock-in mice (Merino *et al.*, 2009), with Bim^{Bad} binding only to Bcl-2, Bcl-x_L and Bcl-w but not Mcl-1 or A1, Bim^{Noxa} only binding Mcl-1 and A1 and, as a control, Bim^{Puma} still binding all pro-survival family members (Figure 1a). Bim^{Bad} as well as Bim^{Noxa} mutant mice had abnormally increased numbers of leukocytes (Merino *et al.*, 2009), although this phenotype was clearly less pronounced compared to Bim-deficient ($Bim^{-/-}$ (Bouillet *et al.*, 1999)) animals. Thus, for optimal induction of developmentally programmed cell death (at least in the hematopoietic system), Bim must be able to interact with all pro-survival Bcl-2 family members. We have exploited these Bim BH3 region replacement mutant mice to investigate the role of Bim's binding specificity in tumor suppression, using the Eμ-*Myc* mouse lymphoma model. These studies demonstrate that for optimal suppression of Myc-induced lymphomagenesis, Bim must be able to bind all Bcl-2-like pro-survival proteins.

Results and Discussion

Bim functions as a tumor suppressor in both mice (Egle *et al.*, 2004) and humans, with loss of both *Bim* alleles or suppression of Bim protein expression detected in mantle cell lymphoma (Tagawa *et al.*, 2005) and certain other B lymphoid malignancies, such as Burkitt Lymphoma (Anderton *et al.*, 2008). The tumor suppressor activity of Bim was first demonstrated in Eμ-*Myc* mice (Egle *et al.*, 2004) where loss of one *Bim* allele shortened median survival from the normal ~100 days to ~77 days and loss of both alleles to 57 days. Although loss of Puma, Bmf, Bad or Noxa can also accelerate Myc-induced lymphomagenesis (Frenzel *et al.*, 2010; Michalak *et al.*, 2009), loss of one allele of these genes did not have significant impact, indicating these BH3-only proteins are less potent tumor suppressors in this context than Bim. These differences between BH3-only proteins could be due to their level of expression, their specific regulation and/or their ability to bind pro-survival Bcl-2 proteins and hence their efficacy to trigger apoptosis.

The *Bim*^{Bad} and *Bim*^{Noxa} mutations both accelerated lymphoma development in Eμ-*Myc* transgenic mice

Although the tumor suppressive activity of Bim has been established, it is not clear whether this requires its ability to bind to all or only a limited subset of the pro-survival Bcl-2 family members. This question could be addressed by crossing Eμ-*Myc* mice with our mutant strains of mice in which Bim has been altered to restrict its binding to Bcl-2, Bcl-x_L and Bcl-w (Bim^{Bad}) or Mcl-1 plus A1 (Bim^{Noxa}). Regardless of the *Bim* genotype, all Eμ-*Myc* transgenic mice developed lymphoma. Western blot analysis demonstrated that Bim^{Bad}, Bim^{Noxa} and Bim^{Puma} mutant proteins were expressed in such tumors at levels comparable to wt Bim (Figure 1b). Moreover, expression of the Bim BH3 exchange mutant proteins did not significantly affect the expression of pro-survival Mcl-1, Bcl-2 and Bcl-x_L in Eμ-*Myc* lymphomas arising in these mutant animals (Figure 1b).

The survival of Eμ-*Myc*/*Bim*^{BH3} mutant mice was compared with the survival of control Eμ-*Myc* mice. Remarkably, a single allele mutation of *Bim* into either *Bim*^{Bad} or *Bim*^{Noxa} significantly decreased the survival of Eμ-*Myc* mice from ~100 to 64 or 70 days, respectively (Figure 2a and c). This median lifespan is similar to that previously reported for Eμ-*Myc*/*Bim*$^{+/-}$ mice (77 days; (Egle *et al.*, 2004)), and consistent with the notion that Bim functions as a haplo-insufficient tumor suppressor. The Eμ-*Myc*/*Bim*$^{Bad/Bad}$ and Eμ-*Myc*/*Bim*$^{Noxa/Noxa}$ mice became sick even more rapidly, with a median survival of 50 and 58 days, respectively (Figure 2c). This reduction in median survival is remarkably similar to the 57 days reported for Eμ-*Myc*/*Bim*$^{-/-}$ mice (Egle *et al.*, 2004). Importantly, all the mice used in the present study were of the same genetic background (C57BL/6) as the mice used in our former study (Egle *et al.*, 2004). Thus, in this model of tumorigenesis, Bim^{Bad} and Bim^{Noxa} proteins behaved as complete loss-of-function mutants of Bim, indicating that in B lymphoid cells undergoing Eμ-*Myc* induced neoplastic transformation, the life-versus-death decision must be very tightly balanced. Since lymphomagenesis is accelerated both by disabling Bim from binding to either Bcl-2, Bcl-x_L and Bcl-w (Bim^{Noxa}) or by preventing its interaction with Mcl-1 plus A1 (Bim^{Bad}), we hypothesize that both of these subgroups of pro-survival Bcl-2 proteins must be critical to sustain pre-leukemic Eμ-*Myc* B lymphoid cells undergoing neoplastic transformation. Consistent with a role for Mcl-1 in such a process, its haplo-insufficiency protects mice from Myc induced AML development (Xiang *et al.*, 2010). As for the relative importance of endogenous expression of Bcl-2, Bcl-x_L or Bcl-w in Eμ-*Myc* induced lymphomagenesis, we predict that Bcl-x_L is most critical, given that loss of Bcl-2 had no impact on this disease (Kelly *et al.*, 2007) and that Bcl-x_L but not Bcl-w is expressed at readily detectable levels in pre-malignant Eμ-*Myc* B lymphoid cells (Michalak *et al.*, 2009).

The *Bim*^{Puma} mutation had no significant impact on lymphoma development in Eμ-*Myc* mice

We have previously shown that the replacement of the BH3 domain of Bim with the BH3 domain of Puma did not impair its ability to bind to all pro-survival Bcl-2-like proteins (Merino *et al.*, 2009). This mutation did, however, cause a significant, albeit relatively minor, increase in leukocytes, indicating that actions of Bim that are independent of its ability to bind to pro-survival Bcl-2 family, such as direct interaction with Bax/Bak, must also be critical for optimal activity in developmentally programmed cell death (at least in the hematopoietic system). Interestingly, the median lifespans of Eμ-*Myc*/*Bim*$^{Puma/+}$ (110 days) and (despite a trend towards accelerated disease) even of the Eμ-*Myc*/*Bim*$^{Puma/Puma}$ mice (84 days) was not significantly different from the median survival of control Eμ-*Myc* mice (Figure 2b and c). This indicates that the actions of Bim that are independent of its ability to bind pro-survival Bcl-2-like proteins are not essential for its tumor suppressive function, at least in Eμ-*Myc* mice. This apparent discrepancy with our previous conclusions (Merino *et*

al., 2009) might simply be due to a difference of experimental setting. None of the cells considered in our former study were malignant, whereas they are in this study. This does, however, not exclude that direct activation of Bax/Bak can play a role in killing of Eμ-*Myc* B lymphoid cells undergoing transformation (Letai *et al.*, 2004), as this function might be accomplished by another BH3-only protein present in these cells (e.g. Puma or Noxa) as long as Bim (i.e. Bim^{Puma} in this case) can neutralize all pro-survival Bcl-2 proteins.

The Bim^{Bad} and Bim^{Noxa} but not the Bim^{Puma} mutation enhanced the severity of Eμ-*Myc* lymphoma

At autopsy, the spleen weights of all Eμ-*Myc*/Bim^{BH3} exchange mutant mice were similar to those of sick control Eμ-*Myc* littermates (Figure 3a). Remarkably, however, the Eμ-*Myc*/$Bim^{Bad/Bad}$ and Eμ-*Myc*/$Bim^{Noxa/Noxa}$ mice had significantly higher white blood cell (WBC) counts (Figure 3b). Conversely, the Eμ-*Myc*/$Bim^{Puma/Puma}$ mice showed similar leukemic burden compared to control Eμ-*Myc* mice (Figure 3b). Finally, immuno-phenotyping did not reveal significant differences between lymphomas from the various genotypes of mice. $B220^{+}IgM^{+}$ B lymphomas were slightly more abundant in the control Eμ-*Myc* mice, whereas $B220^{+}sIgM^{-}$ pre-B lymphomas predominated in all other genotypes (Figure 3c).

In conclusion, this study demonstrates that the binding to pro-survival Bcl-2 family members plays an important role in the tumor suppressor activity of the BH3-only protein Bim. The data also indicate that Mcl-1 and Bcl-x_L may play critical roles in sustaining the survival of pre-leukemic Eμ-*Myc* B lymphoid cells whilst they are acquiring additional oncogenic lesions that promote their progression to full malignancy. This hypothesis could be tested by generating Eμ-*Myc* mice in which Mcl-1 or Bcl-x_L can be deleted specifically in B lymphoid cells. If proven correct, one may consider to target Bcl-x_L and/or Mcl-1 as a strategy for early intervention to prevent or delay Myc-induced tumorigenesis if early detection of pre-malignant lesions is possible or if patients are known to be predestined to develop such tumors.

Acknowledgments

We thank Mikara Robati and Owen Siggs for help with experiments, Jerry Adams, Suzanne Cory, Clare Scott and Cyril Clybouw for insightful discussions and Eμ-*Myc* mice, Bruno Helbert and Carley Young for mouse genotyping, Emily Sutherland and Giovanni Siciliano for mouse husbandry and Jason Corbin for blood sample analysis. This work was supported by the Australian National Health and Medical Research Council (program grant 461221, Australia Fellowship (AS) and Career Development Award (PB)), the National Cancer Institute (CA43540), the Australian Research Council (DM), the Leukemia and Lymphoma Society (LLS Specialized Center of Research grant 7015) and operational infrastructure grants through the Australian Government (IRISS) and the Victorian State Government (OIS).

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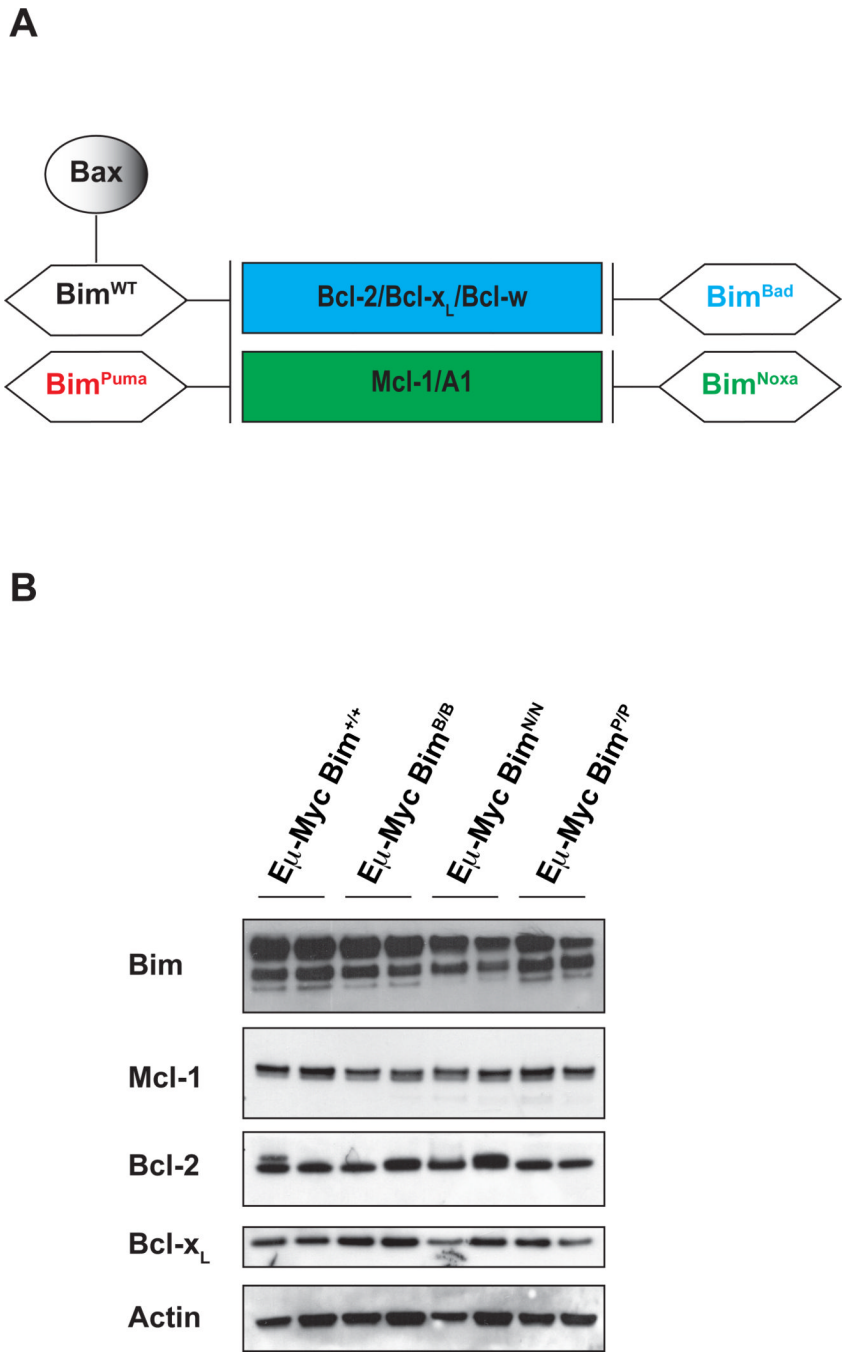
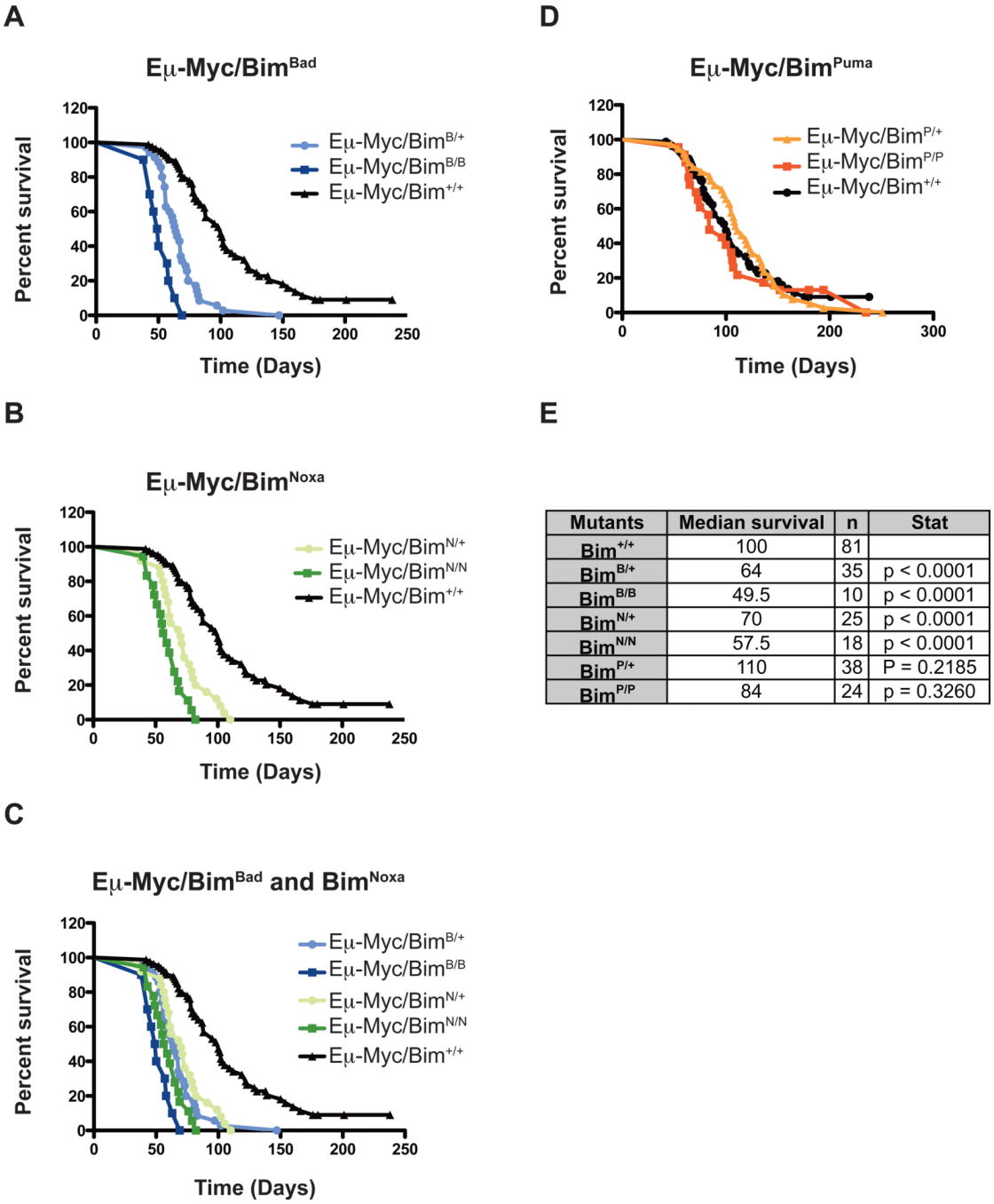


Figure 1. Expression of mutant Bim proteins in Eμ-*Myc* tumors

(a) Schematic representation of specificity of binding of Bim^{BH3} mutants to pro-survival Bcl-2 family members. (b) Western blot analysis to determine expression of Bim^{BH3} mutants, Mcl-1, Bcl-2, Bcl-x_L and Actin (used as loading control) in Eμ-*Myc* lymphomas ($B220^{+}IgM^{-}$) from control Eμ-*Myc* as well as Eμ-*Myc*/$Bim^{Bad/Bad}$, Eμ-*Myc*/$Bim^{Noxa/Noxa}$ and Eμ-*Myc*/$Bim^{Puma/Puma}$ mice (extracts from two different mice for each genotype). Western blotting was carried out by standard procedures, using protein extracts generated by lysis of lymphoma cell suspensions in a buffer containing (20 mM Tris-pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1% Triton-X 100, protease inhibitors MiniComplete, EDTA free, Roche). Proteins were detected using antibodies to Bim (14A8

or 3C5, ENZO), Bcl-2 (BD Pharmingen), Bcl-x_L (BD Bioscience), Mcl-1 (Rockland) or Actin (Sigma). Secondary antibodies were HRP-conjugated and detection used Enhanced Chemoluminescence (ECL, Healthcare).



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Mutants	Median survival	n	Stat
$Bim^{+/+}$	100	81	
$Bim^{B/+}$	64	35	p < 0.0001
$Bim^{B/B}$	49.5	10	p < 0.0001
$Bim^{N/+}$	70	25	p < 0.0001
$Bim^{N/N}$	57.5	18	p < 0.0001
$Bim^{P/+}$	110	38	P = 0.2185
$Bim^{P/P}$	84	24	p = 0.3260

Figure 2. The *Bim*^{Bad} and *Bim*^{Noxa} mutations accelerated Eμ-*Myc* induced lymphomagenesis but the *Bim*^{Puma} mutation had no significant impact

Analysis of lymphoma-free survival was carried out using Graph Pad Prism (version 5.0a). (a) Lymphoma-free survival of control Eμ-*Myc* as well as Eμ-*Myc/Bim*$^{Bad/+}$, Eμ-*Myc/Bim*$^{Bad/Bad}$, Eμ-*Myc/Bim*$^{Noxa/+}$ and Eμ-*Myc/Bim*$^{Noxa/Noxa}$ mice. (b) Lymphoma-free survival of control Eμ-*Myc* as well as Eμ-*Myc/Bim*$^{Puma/+}$ and Eμ-*Myc/Bim*$^{Puma/Puma}$ mice. (c) Summary of data presented in (a) and (b), showing for each genotype of mice the median survival, the number of mice analyzed and statistical analysis of difference in survival in comparison to control Eμ-*Myc* mice. Statistical evaluation of tumor-free survival data was

undertaken using a log-rank test (Mantel-Cox) and 1-way ANOVA test on Kaplan-Meier curves.

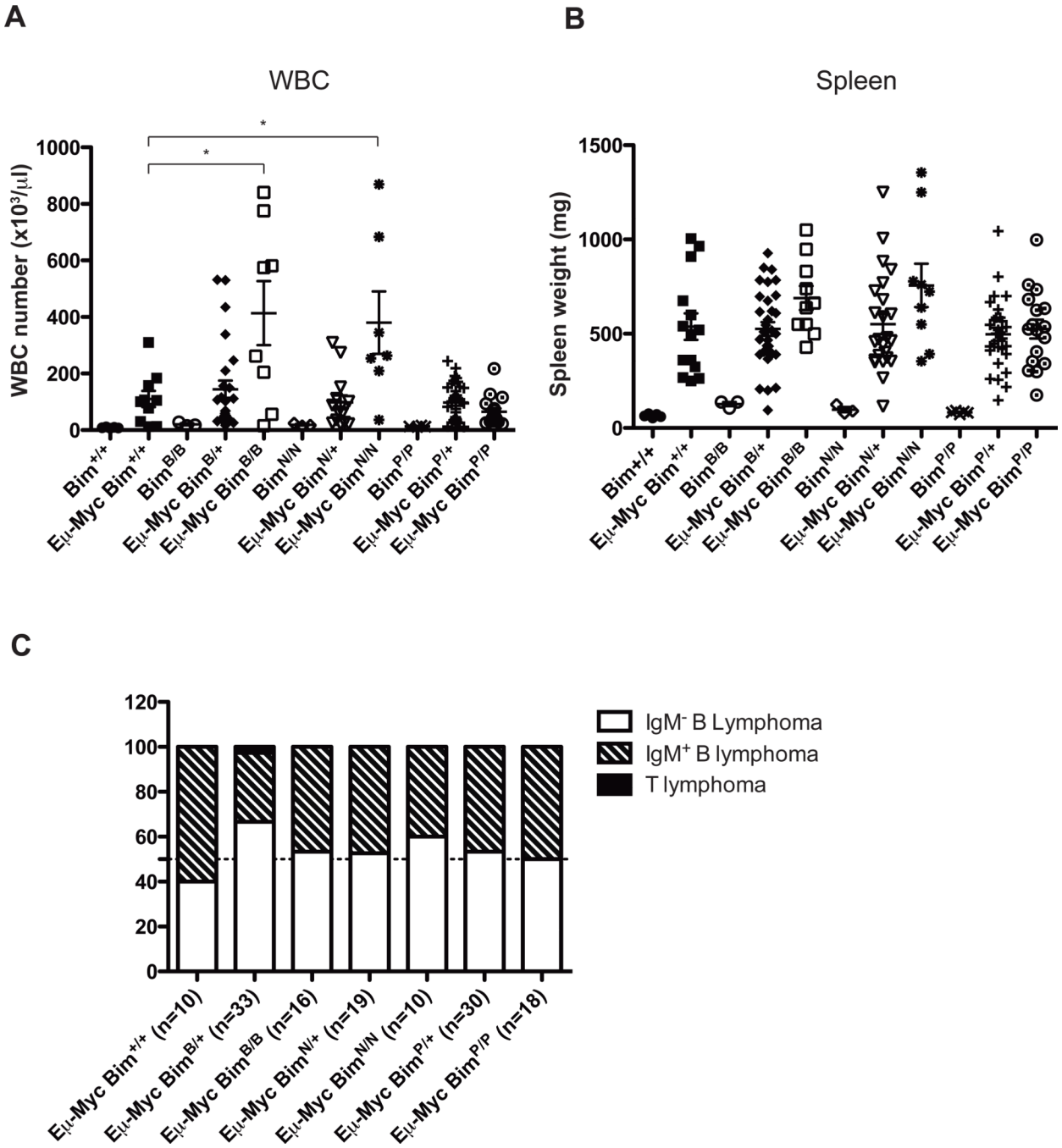

Figure 3. The *Bim*^{Bad} and *Bim*^{Noxa} mutations but not the *Bim*^{Puma} mutation increased the leukemic burden in sick Eμ-*Myc* transgenic mice

(a) Number of WBC and (b) spleen weight of wt (Bim$^{+/+}$), control Eμ-*Myc* (Eμ-*Myc/Bim*$^{+/+}$), *Bim*$^{Bad/Bad}$, Eμ-*Myc/Bim*$^{Bad/+}$, Eμ-*Myc/Bim*$^{Bad/Bad}$, *Bim*$^{Noxa/Noxa}$, Eμ-*Myc/Bim*$^{Noxa/+}$, Eμ-*Myc/Bim*$^{Noxa/Noxa}$, *Bim*$^{Puma/Puma}$, Eμ-*Myc/Bim*$^{Puma/+}$ and Eμ-*Myc/Bim*$^{Puma/Puma}$ mice at autopsy. Peripheral blood was analyzed using the ADVIA hematology analyzer (Bayer). Data represent means+/−SEM (n=numbers of animals analyzed for each genotype). Statistical analysis was performed using the Student t test. Statistically significant differences in comparison to Eμ-*Myc/Bim*$^{+/+}$ mice are indicated (*P<0.05). (c) Diagrammatic representation of the relative frequencies of lymphoma types (sIg^{-}pre-B

lymphoma, sIg^+ B lymphoma or $Thy1^+$ T lymphoma) observed in mice of the indicated genotypes (n= numbers of mice analyzed for each genotype). Sick mice were sacrificed, lymphomas harvested and single cell suspensions prepared for FACS (FacsCalibur2; Becton Dickinson) analysis using fluorochrome-conjugated antibodies against IgM (5.1 or 333.12), IgD (11-26C) and B220 (RA3-6B2).