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Alternative splicing of Bim and Erk-mediated Bim_{EL} phosphorylation are dispensable for hematopoietic homeostasis *in vivo*

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The pro-apoptotic BH3-only protein Bim has a major role in hematopoietic homeostasis, particularly in the lymphocyte compartment, where it strongly affects immune function. The three major Bim isoforms (Bim_{EL}, Bim_L and Bim_S) are generated by alternative splicing. Bim_{EL}, the most abundant isoform, contains a unique sequence that has been reported to be the target of phosphorylation by several MAP kinases. In particular, Erk1/2 has been shown to interact with Bim_{EL} through the DEF2 domain of Bim_{EL} and specifically phosphorylate this isoform, thereby targeting it for ubiquitination and proteasomal degradation. To examine the physiological importance of this mechanism of regulation and of the alternative splicing of Bim, we have generated several Bim knock-in mouse strains and analyzed their hematopoietic system. Although mutation in the DEF2 domain reduces Bim_{EL} degradation in some circumstances, this mutation did not significantly increase Bim's pro-apoptotic activity *in vivo* nor impact on the homeostasis of the hematopoietic system. We also show that Bim_{EL} and Bim_L are interchangeable, and that Bim_S is dispensable for the function of Bim. Hence, we conclude that physiological regulation of Bim relies on mechanisms independent of its alternative splicing or the Erk-dependent phosphorylation of Bim_{EL}.
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Deregulation of apoptosis contributes to a broad range of diseases, including autoimmunity, cancer and degenerative disorders.[1,2] The Bcl-2 family members, classified as pro-survival or pro-apoptotic proteins, are key components of the mitochondrial (also called 'intrinsic' or 'Bcl-2-regulated') apoptotic pathway.[3] Pro-survival proteins (e.g. Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1) are the guardians of mitochondrial integrity and their overexpression prevents cell death induced by diverse cytotoxic stimuli, including cytokine deprivation and many forms of intracellular damage. BH3-only proteins are transcriptionally and/or post-translationally activated by cytotoxic stimuli and their activation leads to the activation of Bax and Bak and the subsequent induction of cell death.[4] Cell survival is regulated by the tight balance between pro-survival proteins and the expression level as well as the activation state of the BH3-only proteins.[3]

The BH3-only protein Bim is a major regulator of immune homeostasis and imposes a critical barrier against autoimmune disease and tumor development.[5] Moreover, deregulation of Bim due to homozygous deletion of its gene, epigenetic regulation of its promoter or phosphorylation-mediated proteasomal degradation of its protein, has been associated with reduced apoptosis and accelerated tumor development.[6–9] Downregulation of Bim expression also triggers resistance to various chemotherapeutic agents, for example, Imatinib, Paclitaxel or Bortezomib.[10–13]

Three major isoforms of Bim are produced by alternative splicing: Bim_{EL}, Bim_L and Bim_S.[14] Bim_{EL} differs from Bim_L by the inclusion of exon 3, an unusual exon that has characteristics of an intron (GT/AG boundaries, branch point and polypyrimidine tract).[15] (Figure 1A). When overexpressed, the three Bim isoforms appear to have different pro-apoptotic potential, Bim_S being the most potent killer and Bim_{EL} the weakest.[16,17] However, whether any isoform has a specific role is not known. Interestingly, the extra sequence in Bim_{EL} (amino acids 42–98 in mouse Bim_{EL}) has been shown to contain residues critical for the post-translational regulation of Bim protein stability. Activation of the MEK/Erk pathway has been shown to prime Bim_{EL} for ubiquitination and proteasomal degradation in various cell types, including normal as well as transformed lymphocytes, osteoclasts, MEFs and cultured cell lines.[18–22] Moreover, it has been reported that oncogenic protein kinases, such as mutant B-Raf or N-Ras, can cause a reduction in Bim protein levels through Erk1/2 activation, thereby promoting tumor development and chemoresistance of cancer cells.[9,10,23] Hence, activating Bim, for example by

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Abbreviations: MAP, mitogen-activated protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMA, Phorbol 12-myristate 13-acetate; FACS, fluorescence activated cell sorting; JNK, c-Jun N-terminal kinases; ERK, extracellular signal-regulated kinases; 3′UTR, 3′ untranslated region; EGTA, ethylene glycol tetraacetic acid; ECL, enhanced chemiluminescence; HRP, horseradish peroxydase
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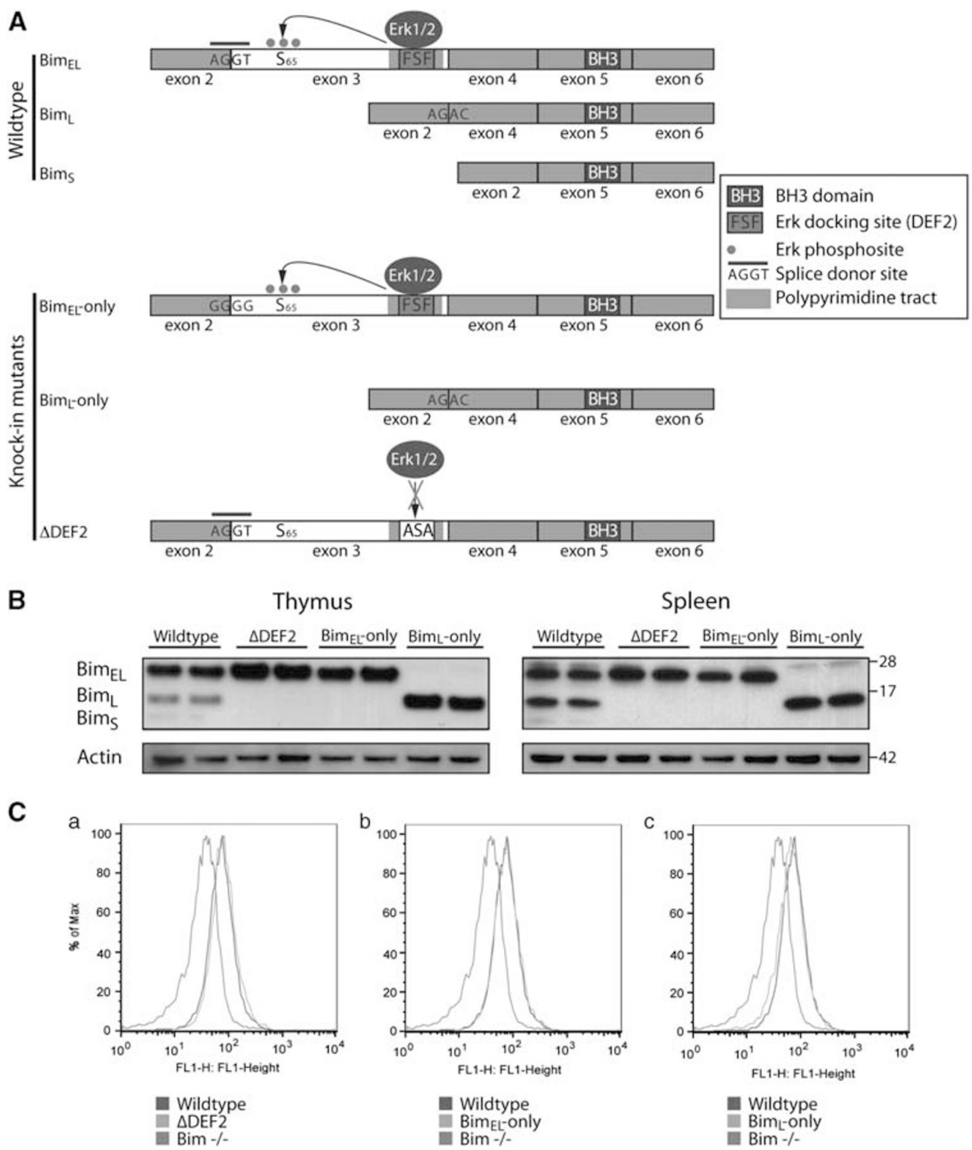

Figure 1 Generation of Bim knock-in mice. (**A**) Schematic representation of how Erk1/2-mediated phosphorylation is thought to regulate the pro-apoptotic activity of Bim_{EL}. Activated Erk1/2 kinase docks on the DEF2 motif (FSF) and phosphorylates Bim_{EL} on three positions (S55/65/73 in mouse; S59/S69/S77 in human). The white box denotes the sequences encoded by exon 3. Note the intron-like structure of exon 3, with the AG/GT splice donor site that is mutated in Bim_{EL}-only mutant mice, as well as the presence of the DEF2 motif in the poly-pyrimidine tract (light-red box) preceding the splice acceptor site at the end of this exon. (**B**) Expression of Bim and β-actin (loading control) was analyzed by western blotting of lysates from splenocytes and thymocytes of two mice of each genotype. (**C**) Intracellular immunostaining of Bim was performed in thymocytes of wild-type, ΔDEF2 (a), Bim_{EL}-only (b) and Bim_{L}-only (c) mice, and analyzed by flow cytometry. *Bim*$^{-/-}$ mice were used as a negative control. The color reproduction of this figure is available at the *Cell Death and Differentiation* journal online

impeding its rapid degradation, has been proposed as a strategy to kill tumor cells in which the Ras/Raf/Erk1/2 pathway is constitutively activated.[9,10,23]

In vitro experiments showed that the Erk1/2 kinase interacts with Bim_{EL} through a domain termed DEF2 specific to that isoform[18] and phosphorylates it at three serine residues, including S65 (S69 in human Bim_{EL})[18,20,21] (Figure 1A). Mutation of this domain in Bim_{EL} ('ΔDEF2 mutant') inhibited Erk1/2-dependent phosphorylation and proteasomal degradation of Bim_{EL} *in vitro*, and thereby increased its pro-apoptotic potency, at least in overexpression systems.[18]

To evaluate the physiological importance of the alternative splicing of Bim and specifically of Erk-mediated regulation of Bim_{EL}, we have generated knock-in mice expressing only Bim_{EL} (Bim_{EL}-only mice), only Bim_{L} (Bim_{L}-only), or only a mutant form of Bim_{EL} with a mutation in the DEF2 domain (ΔDEF2). By comparing them with wild-type mice, we find that Bim_{EL} and Bim_{L} are interchangeable, and that lack of Erk1/2-mediated phosphorylation does not increase Bim's pro-apoptotic function *in vivo*. Moreover, as none of the ΔDEF2, Bim_{EL}-only or Bim_{L}-only mice express the Bim_{S} isoform and are indistinguishable from wild-type mice, we also conclude that expression of Bim_S is not necessary for full function of Bim *in vivo*. Thus, the results suggest that processes other than alternative splicing or Erk1/2-mediated phosphorylation of Bim_{EL} must be critical for regulating the pro-apoptotic activity of Bim physiologically.

Results

Generation of Bim knock-in mutant strains of mice. Mice that can only produce Bim_{EL} (Bim_{EL}-only mice) were obtained by mutating the splice donor site (AG/GT) located between exons 2 and 3 into a non-spliceable sequence (GGGG) (Figure 1A). Mice that can only produce Bim_L (Bim_L-only mice) were generated by deleting exon 3 from the genomic DNA.[24] Note that since the sequence of the junction between exons 2 and 4 (AGAC) cannot be used as a splice donor site, these mice are also incapable of making Bim_S, which requires a splice between exons 2 and 5.[15] Mice producing Bim_{EL} that cannot interact with Erk1/2 (ΔDEF2 mice) were generated by mutating the Bim coding region to change the amino-acid sequence $F_{93}S_{94}F_{95}$ into $A_{93}S_{94}A_{95}$ (Figure 1A). Since the sequence encoding the FSF motif (TTCTCTTTT) forms part of the poly-pyrimidine tract preceding the slice acceptor site in exon 3, this mutation (GCTTCTGCT) was also expected to prevent the splicing of exon 3 and thus, preclude the expression of Bim_L and Bim_S (Supplementary Figure S1a). Mice of all three mutant strains were fertile, and outwardly indistinguishable from wild-type animals.

The Bim isoforms produced in the various mice were analyzed by western blot analysis of thymocytes and splenocytes. Bim_{EL} was the most abundant isoform in wild-type mice, followed by Bim_L, whereas Bim_S was barely detectable (Figure 1B). As designed, Bim_{EL}-only and Bim_L-only mice expressed exclusively Bim_{EL} or Bim_L (Figure 1B). As predicted, ΔDEF2 mice expressed only Bim_{EL}, demonstrating that the mutated polypyrimidine tract did indeed impair the splicing of exon 3 and hence prevented the expression of Bim_L and Bim_S protein (Figure 1B and Supplementary Figure S1a) and mRNA (Supplementary Figure S1b). The ΔDEF2 strain is therefore directly comparable to the Bim_{EL}-only strain.

To ascertain whether the total amount of Bim protein expressed in the various mutant mouse strains was comparable to that of wild-type mice, we performed intracellular staining of thymocytes with an anti-Bim antibody that recognizes all Bim isoforms (3C5).[25] Bim-deficient thymocytes were used as a negative control (red line). No significant difference was detected in the overall amount of Bim expressed between thymocytes from the knock-in mutant mice and those from wild-type mice (Figure 1C).

Expression of Bcl-2 family member and Bim partners. The phosphorylation status of Bim_{EL} has been reported to modulate its binding to Bcl-x_L and Mcl-1.[26] As the binding of Bim to the pro-survival Bcl-2-like proteins affects its turnover[27,28] (Merino *et al*., submitted), we assessed the expression level of other Bcl-2 family members in thymocytes and splenocytes of the mutant strains of mice (Figure 2a). The levels of the Bcl-2 family members Bcl-2, Bcl-x_L, Mcl-1, Bmf, Bax and Bak, did not differ between the Bim mutant strains and wild-type mice (Figure 2a).

We also performed co-immunoprecipitation studies to test whether the mutations introduced in our mutant mice could modify the interactions of Bim with its partners. As expected from the Bim intracellular immunostaining (Figure 1C), equal amounts of Bim were immunoprecipitated from lysates of thymocytes and splenocytes from all the strains (Figure 2b). Importantly, similar amounts of Bcl-2, Bcl-x_L and Mcl-1 co-immunoprecipitated with Bim from cells of the different mutant strains and the wild-type mice. Altogether, these data show that the mutations introduced into Bim did not change the level of expression of other Bcl-2 family members, the total level of Bim or its ability to bind pro-survival Bcl-2-like proteins.

Bim phosphorylation and degradation are impaired in ΔDEF2 and Bim_L-only mutant mice. Consistent with previous reports,[25,29] mitogenic activation of wild-type T cells by PMA/ionomycin rapidly induces phosphorylation of Bim_{EL}, as evidenced by its reduced electrophoretic mobility 1 h after stimulation (Figure 3a). This process was, as expected,[25,29] associated with the activation of Erk1/2. As previously shown,[25,29] treatment with an inhibitor of the Erk pathway, U0126, inhibited the PMA/ionomycin-induced activation of Erk1/2, as well as the phosphorylation of Bim_{EL} in wild-type T cells (Figure 3a). In order to verify that the ΔDEF2 mutation impaired Erk1/2-mediated phosphorylation of Bim_{EL}, purified T lymphocytes were stimulated for 1 h with PMA/ionomycin and cell lysates were analyzed by two-dimension (2D) electrophoresis (Figure 3b).

Similar profiles of phospho-isomers of Bim_{EL}, all falling within a pH isoelectric (pI) range 5–7, were detected in unstimulated mature T cells from wild-type, ΔDEF2 and Bim_{EL}-only mice. Mitogenic activation of wild-type and Bim_{EL}-only T cells induced a dramatic shift of most Bim_{EL} spots to a more negative, that is, acidic, PI (range 4–5; Figure 3b), consistent with phosphorylation of Bim_{EL} at additional sites. In contrast, PMA/ionomycin stimulation of ΔDEF2T cells induced only a minor shift of Bim_{EL} spots, with several spots remaining within the 5–7 pI range (Figure 3b). These results show that the ΔDEF2 mutation inhibited phosphorylation of Bim_{EL} on at least two sites, consistent with a previous *in vitro* study.[18] PMA/ionomycin treatment, however, did not alter the profile of Bim_L phospho-isomers (Figure 3b), demonstrating that this stimulus does not promote Bim_L phosphorylation.

Erk1/2-mediated phosphorylation primes Bim_{EL} for ubiquitination and proteasomal degradation.[18–22] Indeed, in wild-type and Bim_{EL}-only T cells, phosphorylation of Bim_{EL} (evident by slower migration on SDS-PAGE) after 2 h of mitogenic stimulation was associated with a significant decrease in Bim_{EL} levels at both 2 and 24 h of stimulation (Figure 3c). In contrast, little if any electromobility shift of Bim was evident in PMA/ionomycin-stimulated T cells from ΔDEF2 and Bim_L-only mice (Figure 3c), indicating that Bim phosphorylation was unaffected, consistent with the 2D experiments (Figure 3b). Moreover, their Bim level did not diminish significantly over the 24 h of treatment (Figure 3c). These experiments showed that Bim_{EL} phosphorylation and the

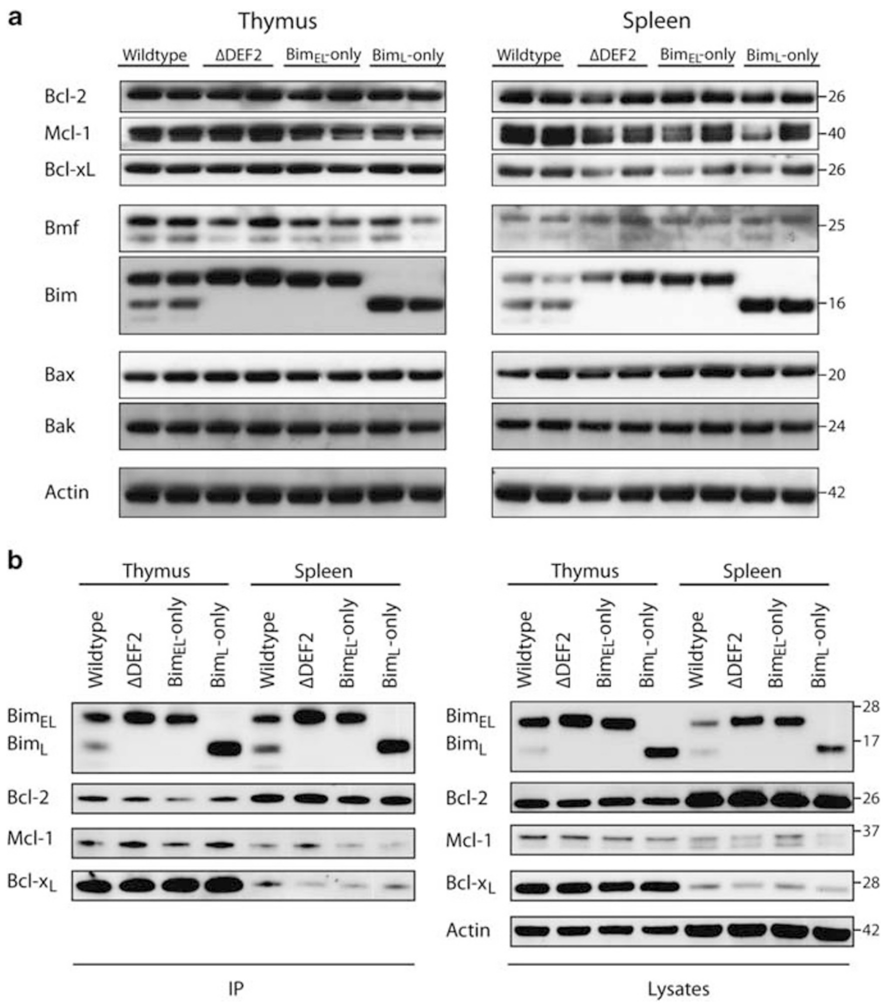

Figure 2 Expression of Bcl-2 proteins and Bim partners in wild-type, ΔDEF2, BimEL-only and BimL-only mice. (**a**) Expression of the pro-survival Bcl-2 family members Bcl-2, Bcl-x_L and Mcl-1, BH3-only proteins Bim, Bmf and the multi-BH domain containing pro-apoptotic Bcl-2 family members Bax and Bak was analyzed by western blotting of lysates from splenocytes and thymocytes of two mice of each genotype. β-actin was used as a loading control. (**b**) Interaction between Bim and Bcl-2, Bcl-x_L and Mcl-1 was studied by co-immunoprecipitating Bim from protein extracts of thymocytes and splenocytes of the indicated mice

consequent proteasomal degradation were inhibited in ΔDEF2 mice and that Bim_L is not a target of Erk1/2-mediated phosphorylation.

Normal hematopoietic system in Bim_{EL}-only, Bim_L-only and ΔDEF2 mice. Loss or reduction of Bim function profoundly affects lymphoid cell development.[5] Bim-deficient mice have increased numbers of white blood cells and splenocytes.[5] Moreover, mutations in the BH3 domain of Bim that confine its binding to certain pro-survival Bcl-2 family members also elevates white blood cells and splenocytes.[30] Therefore, we enumerated the white blood cells (Figure 4a), thymocytes (Figure 4b) and splenocytes (Figure 4c) of the wild-type and mutant mice. No significant differences were observed between these strains (Figures 4a-c). Moreover, in the blood of all three Bim mutant mice, the percentages of lymphocytes (Supplementary Figure S2a) and neutrophils (Supplementary Figure S2b) and the numbers of platelets (Supplementary Figure S2c) were similar to those in wild-type mice. The representation of B- and T-cell types in lymph nodes (Supplementary Figure S2d) and spleen (Supplementary Figure S2f), as well as the T-cell subset composition in the thymus (Supplementary Figure S2e), were also normal in all the knock-in mouse strains. These results demonstrate that the mutations that we introduced into the *Bim* gene did not significantly alter its physiological function.

Sensitivity of DP thymocytes to different apoptotic stimuli. Bim is critical for the apoptosis of lymphocytes induced by diverse cytotoxic stimuli, including cytokine withdrawal as well as treatment with ionomycin, dexamethasone or etoposide.[5] Experiments using overexpression systems *in vitro* have indicated that mutation of the DEF2 region increases the pro-apoptotic activity of Bim.[18] If this were relevant to physiological levels of Bim expression, lymphoid cells from Bim-ΔDEF2 mice should die more rapidly than their wild-type counterparts. To test this prediction, we FACS-sorted DP thymocytes from the various strains and cultured them in simple medium

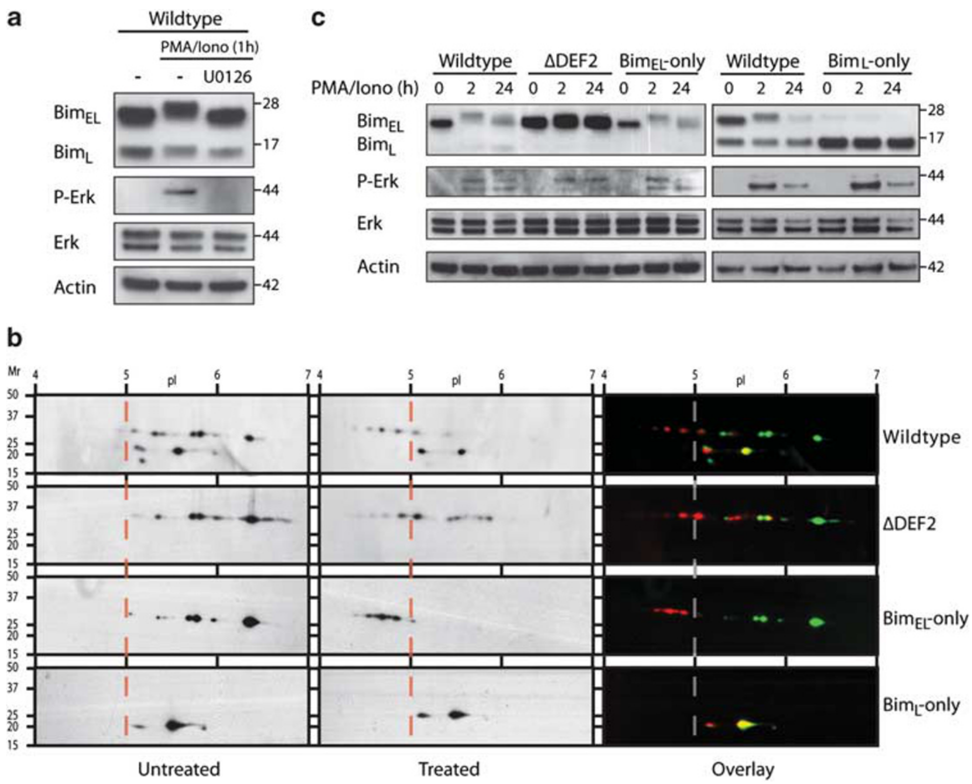

Figure 3 Bim phosphorylation and degradation in Bim-mutant T lymphocytes. (**a**) Expression of Bim, phospho-Erk1/2, Erk1/2 and β-actin (loading control), as assessed by western blotting, in purified T lymphocytes (2×10^6 cells/ml) from wild-type mice that were left untreated or stimulated for 1 h with PMA (2 ng/ml) plus ionomycin (0.1 μg/ml) in medium containing a saturating concentration of recombinant mouse IL-2 in the presence or absence of the MEK1/2 inhibitor U0126 (20 μM). (**b**) Bim-phosphorylated forms revealed by 2D gel electrophoresis in purified T lymphocytes of the indicated strains of mice, which had been left untreated or stimulated (at 2×10^6 cells/ml) for 1 h as described above. Cell lysates were resolved by isoelectric focusing (pI range 4-7) in the horizontal dimension and size fractionation by SDS-PAGE in the vertical dimension. An overlay (yellow) of the immunoblots revealing Bim isoforms in untreated (green) and treated (red) cells was generated by using Adobe Photoshop CS4 software to highlight changes in the relative abundance of multiply phosphorylated Bim isoforms (pI <5, broken line). (**c**) Purified T lymphocytes (2×10^6 cells/ml) from wild-type, ΔDEF2, Bim_{EL}-only and Bim_L-only mice were treated as described above for 0, 2 or 24 h. Expression of Bim, Phospho-Erk1/2, Erk1/2 and β-actin was assessed by western blotting

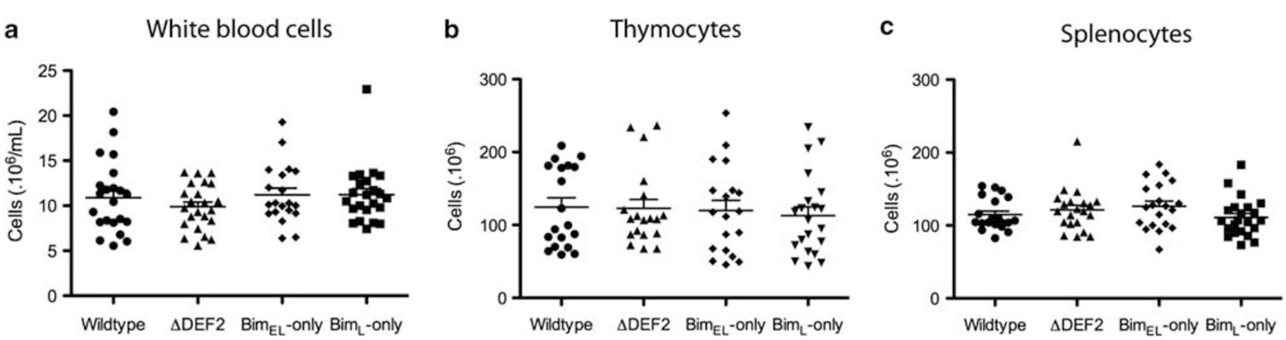

Figure 4 Analysis of hematopoietic cell composition of Bim mutant mice. (**a**) The numbers of white blood cells in wild-type, ΔDEF2, Bim_{EL}-only and Bim_L-only mice were determined using an ADVIA blood analyzer. Data represent mean (± S.E.M.) of at least $n=20$ per genotype ($P>0.05$). (**b** and **c**) The numbers of thymocytes (**b**) and splenocytes (**c**) from wild-type, ΔDEF2, Bim_{EL}-only and Bim_L-only mice were determined by using a Casy cell counter (Schärfe System GmbH, Reutlingen, Germany). Data represent mean (± S.E.M.) of at least $n=19$ per genotype ($P>0.05$)

(i.e. cytokine withdrawal) (Figure 5a) or in presence of ionomycin (Figure 5b), dexamethasone (Figure 5c), etoposide (Figure 5d), PMA (Supplementary Figure S3a), taxol (Supplementary Figure S3b) or the BH3 mimetic ABT-737 (Supplementary Figure S3c), and measured cell survival at the indicated times and concentrations. With all these stimuli, thymocyte survival was comparable for mice of the different genotypes. Thus, the ΔDEF2 mutation or deletion of exon 3 did not augment the pro-apoptotic activity of Bim in these cells.

Bim mutations have no impact on mitogenic activation of T and B lymphocytes. Phosphorylation and degradation of Bim_{EL} have been observed during mitogenic stimulation of

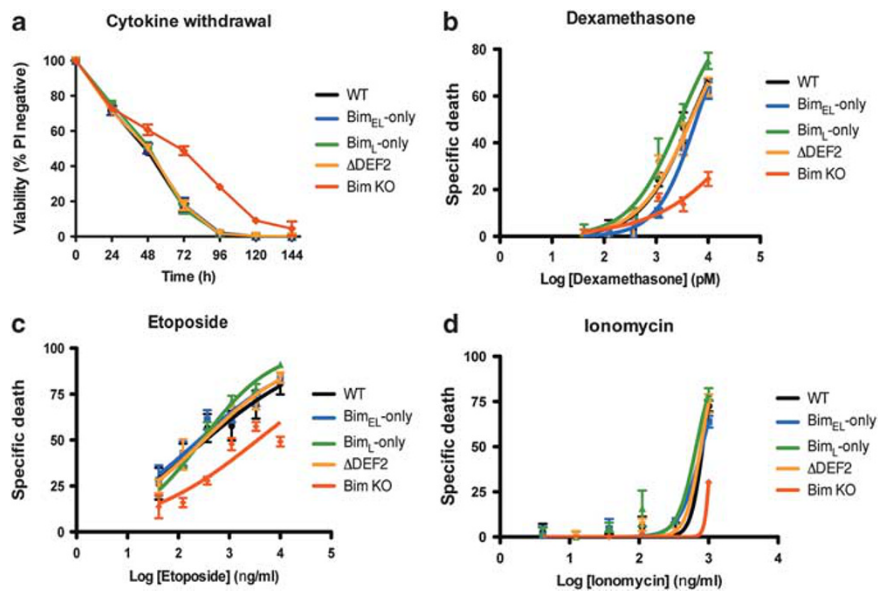

Figure 5 Sensitivity of thymocytes to different apoptotic stimuli. (**a**) CD4$^+$CD8$^+$ thymocytes from wild-type (WT), ΔDEF2, Bim_{EL}-only, Bim_L-only and $Bim^{-/-}$ mice were FACS-sorted and cultured in simple medium (cytokine withdrawal). Total thymocytes from wild-type, ΔDEF2, Bim_{EL}-only, Bim_L-only and $Bim^{-/-}$ mice were cultured in simple medium plus the indicated concentrations of dexamethasone (**b**), etoposide (**c**) or ionomycin (**d**) for 24 h. Cell survival was assessed by PI staining and FACS analysis. Specific death was calculated using the following equation: ((% apoptosis−% spontaneous apoptosis)/(100−% spontaneous apoptosis)). Data represent mean (±S.E.M.) of $n=3$ independent mice per genotype

T and B lymphocytes *in vitro* and proposed to be essential for their survival.[25,29] Indeed, treatment with the Erk1/2 inhibitor UO126 not only blocked Bim_{EL} phosphorylation and proteasomal degradation but also substantially increased the apoptosis of mitogenically activated T and B cells from wild-type but not Bim-deficient mice.[25,29]

As Erk1/2-mediated Bim phosphorylation and degradation were inhibited in lymphocytes from the ΔDEF2 and Bim_L-only mutants (Figures 3b and c), we hypothesized that these cells would exhibit defects in activation. T lymphocytes were purified from lymph nodes and spleen, cultured in the presence of IL-2 and activated by PMA/ionomycin or plate-bounded anti-CD3/CD28 monoclonal antibodies. The percentages of activated, proliferating T cells (CD25$^+$FSC^{hi} Figure 6a) and survival (PI$^-$; Figure 6b) were determined at 24, 48 and 120 h of mitogenic stimulation. Surprisingly, purified ΔDEF2 and Bim_L-only T cells, in which Bim phosphorylation and degradation are impaired (Figure 3c), were activated to the same extent as wild-type or Bim_{EL}-only T cells after 24 h of treatment with PMA/ionomycin (Figure 6a). No significant differences were found at any time point of stimulation (Figure 6a), regardless of whether cells were activated by PMA/ionomycin or anti-CD3/CD28 antibodies (Figure 6a). Moreover, we observed no significant increase in spontaneous apoptosis of quiescent T cells or of the apoptosis associated with mitogen activation of proliferating T cells from any of our mutant mice (Figure 6b). T cells from the wild-type, ΔDEF2, Bim_{EL}-only and Bim_L -only mice all proliferated at the same rate (Supplementary Figure S4).

We tested B-cell activation with purified splenic B cells from the different strains treated with anti-IgM plus anti-CD40 antibodies in the presence of IL-2, IL-4 and IL-5. The percentage and number of activated, proliferating B cells (CD25$^+$FSC^{hi} Supplementary Figure S5a and S5c, respectively) and viable B cells (Supplementary Figures S5b and S5d, respectively) were determined at 24, 48 and 120 h of mitogenic stimulation. As for the T cells, no significant difference in the activation or survival of B cells was observed between the different genotypes.

Collectively, these results demonstrated that inhibition of Erk1/2-mediated Bim degradation did not modify the amplitude and time course of mitogenic activation of T and B cells *in vitro*.

Discussion

Various regulatory mechanisms have been shown to control Bim expression or its activity, both at the transcriptional and post-transcriptional levels.[31] For example, phosphorylation of Bim by various kinases, including Erk1/2, p38, JNK or Akt, occurring mainly within sequences that are unique to Bim_{EL} (encoded by exon 3), have been shown to increase or decrease the stability and consequently pro-apoptotic function of Bim.[20,32,33]

In this study, we generated three novel knock-in mutant strains of mice to evaluate the importance of the sequences encoded by exon 3, which were reported to be critical for its interaction with Erk1/2 in the function of Bim. In particular, we focused on the role of Erk1/2-mediated Bim phosphorylation on the homeostasis of the hematopoietic system, in which Bim has been shown to have an essential and non-redundant role.[5]

Among the three majors isoforms generated by alternative splicing,[16] only the most abundantly expressed, Bim_{EL}, has been reported to be regulated by Erk1/2-mediated phosphorylation.[18,20,21,25,29,34] Indeed, in contrast to Bim_L and Bim_S,

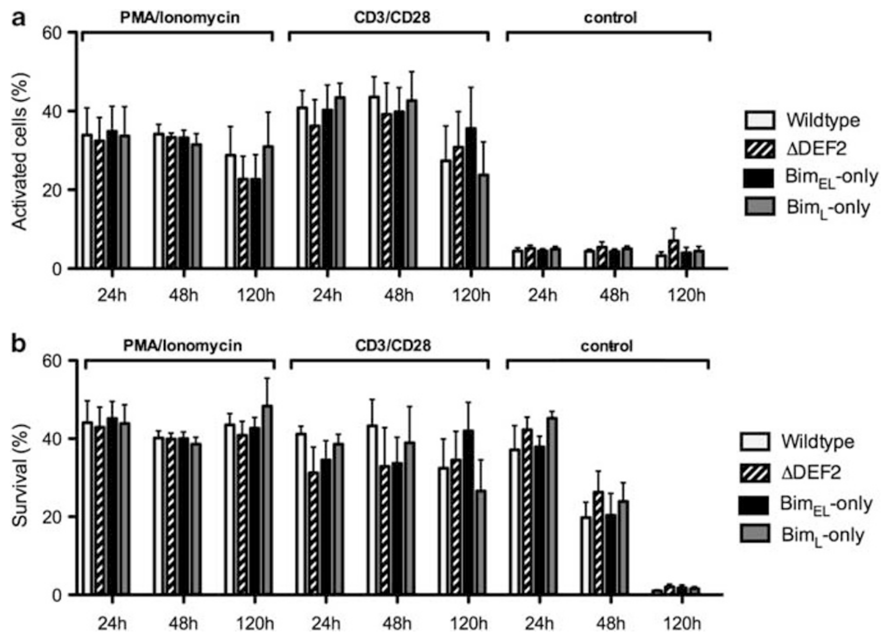

Figure 6 *In vitro* activation of wild-type, ΔDEF2, BimEL-only and BimL-only mice T lymphocytes. (**a** and **b**) Purified T lymphocytes (2×10^6 cells/ml) from wild-type, ΔDEF2, Bim$_{EL}$-only and Bim$_L$-only mice were left untreated or stimulated with PMA (2 ng/ml) plus ionomycin (0.1 μg/ml) or plate-bound monoclonal antibodies to CD3 (10 μg/ml) and CD28 (10 μg/ml) in medium containing saturating concentration of recombinant mouse IL-2. The percentages of activated ($CD25^+FSC^{hi}$) (**a**) and surviving (PI^-FSC^{hi}) (**b**) T cells were measured after 24, 48 and 120 h of culture. Data represent mean (± S.E.M.) of at least 5 mice per genotype ($P > 0.05$)

Bim$_{EL}$ contains within the sequences encoded by exon 3 a DEF-type Erk1/2 docking domain (DEF2), as well as three Erk1/2 phosphorylation sites (S55/65/73 in mouse, S59/S69/S77 in human). Studies performed *in vitro* using mutant forms of Bim have shown that the DEF2 domain and these Erk1/2 phosphorylation sites are necessary for Erk1/2-mediated phosphorylation of Bim and consequent proteasomal degradation.[18,20,21] Our results are consistent with some of these findings, as we did not observe any phosphorylation of Bim$_L$ in mitogenically activated T lymphocytes from Bim$_L$-only mice and T cells from ΔDEF2-Bim$_{EL}$ mice exhibited considerably less phosphorylation than wild-type Bim$_{EL}$ (Figure 3b). Moreover, in mitogenically activated T lymphocytes the degradation of ΔDEF2-Bim$_{EL}$, which is mediated by the proteasome, was considerably reduced compared to wild-type Bim (Figure 3c). Phosphorylation-defective Bim$_{EL}$ mutants (ΔDEF2, S65A or S65G) have been reported to exhibit enhanced killing ability compared with wild-type Bim$_{EL}$, but that study employed overexpression systems in transformed cell lines.[18,20,21,34] It has been also reported that MEFs from mice harboring mutations of the Erk1/2 phosphorylation sites S55, S65 and S73 (Bim^{3SA} mice) or from mice expressing only the Bim$_L$ isoform (BimΔEL) are more sensitive to serum starvation-induced apoptosis than wild-type MEFs.[24]

In contrast to these reports, our findings indicate that, when expressed at physiological levels, ΔDEF2-Bim$_{EL}$ or Bim$_L$ do not possess enhanced killing activity compared with wild-type Bim$_{EL}$, as indicated by the normal composition of the hematopoietic system in ΔDEF2 and Bim$_L$-only mice (Figure 4 and Supplementary Figure S2). These findings indicate that Erk1/2-mediated phosphorylation and other post-translational events associated with the sequences mutated in exon 3 of Bim are not critical for the development and homeostasis of hematopoietic cells. Moreover, thymocytes from ΔDEF2 and Bim$_L$-only mice died at the same rate as wild-type thymocytes when exposed to various cytotoxic stimuli known to require Bim[5] (Figure 5 and Supplementary Figure S3). Thus, Erk1/2-mediated phosphorylation of Bim does not constitute a critical regulatory mechanism for apoptosis induction in this context.

Erk1/2 phosphorylation and degradation of Bim$_{EL}$ have also been shown to be important for the survival of T and B lymphocytes after mitogenic activation *in vitro*.[25,29] Our studies, however, found no defect in the survival, activation and proliferation of mitogenically stimulated T and B cells from ΔDEF2 mice (Figure 6, Supplementary Figures S4 and S5), even though the mutation clearly prevented ERK1/2-mediated phosphorylation and consequent proteasomal degradation of Bim$_{EL}$ in these cells (Figures 3b and c). As the Erk1/2 kinases are known to be active in diverse cellular pathways (in addition to phosphorylation of Bim),[35] loss of Erk1/2 or treatment with U0126 may have caused unrecognized events leading indirectly to Bim-dependent apoptosis induction. This could explain why loss of Bim decreased cell death induced by loss of ERK2[29] or treatment with UO126,[25] as the ΔDEF2 mutation only prevents phosphorylation of Bim$_{EL}$ by Erk1/2 but does not impair any of the other pro-survival pathways that can be activated by these kinases. These include the transcriptional control of the expression of Bcl-2, Bcl-x$_L$ or Mcl-1, or the repression of BH3-only genes such as Bmf. Our present results indicate that Erk1/2-mediated phosphorylation and consequent proteasomal degradation of Bim$_{EL}$ does not constitute a critical mechanism for apoptosis regulation, at least within the hematopoietic system. Our ΔDEF2 mutant mice, which will be freely available, will open unique approaches to test the importance of this process in other cell types.

Besides phosphorylation by Erk1/2, additional modes of regulation, such as direct phosphorylation by the kinases p38,[36] JNK[37] or Akt,[33] targeting the sequences encoded by exon 3 (specific to Bim_{EL}) have been proposed to control the function of Bim_{EL}. Depending on the cell type and phosphorylation sites involved, these phosphorylation events have been reported to either decrease or increase the pro-apoptotic activity of Bim by modifying its stability and/or its binding to other proteins, such as Mcl-1, Bcl-x_L, 14.3.3 or Pin1.[18,20,32,33,36,37] However, our side-by-side comparison of ΔDEF2, Bim_{EL}-only and Bim_L-only mice showed no difference in the composition of their hematopoietic system or the survival of their lymphocytes in culture. These results indicate that Bim_{EL} and Bim_L are interchangeable and hence that none of the modes of regulation targeting the sequence encoded by exon 3 are critical for the control of Bim function *in vivo*. Interestingly, a recent report indicates that the MEK/ERK1/2 signaling pathway regulates the pro-apoptotic activity of Bim by effects on the 3′ UTR of its mRNA in sympathetic neurons.[38] Our results do not exclude that Bim could be regulated by ERK1/2 at the transcriptional level rather than the widely reported post-translational processes involving phosphorylation of Bim_{EL}.[38] Since in all these mice, the mutated forms of BIM have been present from earliest development, it is also possible that adaptation occurred that obscured the effects of the mutations. We may thus imagine that the acute deletion of phosphorylation sites in Bim might have more obvious consequences.

Bim_S has been reported to be the most potently pro-apoptotic isoform of Bim.[16,17] This has been ascribed to its ability to bind to Bax as detected by co-immunoprecipitation of overexpressed proteins, whereas Bim_L and Bim_{EL} do not bind Bax under these conditions.[30,39] The relevance of this Bim_S-Bax interaction for developmentally programmed cell death *in vivo* is not clear. Indeed, we demonstrate here in three different knock-in strains of mice (ΔDEF2, Bim_{EL}-only and Bim_L-only) that the lack of Bim_S expression is not associated with any measurable defect in the function of Bim in hematopoietic homeostasis and apoptosis of thymocytes or activated T cells induced by a broad range of cytotoxic stimuli *in vitro*.

In conclusion, we showed that the inhibition of Erk1/2-mediated phosphorylation and degradation of Bim_{EL} in ΔDEF2 mice does not affect the function of Bim *in vivo*. Moreover, by comparing mice expressing only the isoforms Bim_{EL} or Bim_L, we show that these two isoforms are interchangeable *in vivo* indicating that regulation associated with exon 3 is not critical in this context. Finally, as none of our knock-in mice express Bim_S but all are indistinguishable from wild-type mice, we conclude that any specific function of the Bim_S isoform is dispensable *in vivo*.

Materials and Methods

Mice. ΔDEF2 and Bim_{EL}-only knock-in mice were generated at our Institute using homologous recombination in C57BL/6-derived ES cells, as previously described.[30] Bim_L-only mice, originally described as $Bimdelta_{EL}$ strain,[24] were backcrossed onto a C57BL/6 genetic background. $Bim^{-/-}$ mice were described previously.[5] Peripheral blood erythrocytes and leukocytes were enumerated using an ADVIA hematology system (Bayer, Tarrytown, NY, USA). All animal experiments followed the guidelines of the Melbourne Directorate Animal Ethics Committee.

ΔDEF2 and Bim_{EL}-only mice were genotyped by PCR using the following primers: 5′-GAGAAGGTGGACAATTGCAG-3′ and 5′-AACCAACTGTACCTTGGC TATA-3′.

Western blotting, 2D gel analysis and immunoprecipitation. Cells were lysed in 20 mM Tris pH 7.4, 135 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol and 1% Triton X100 supplemented with complete protease cocktail inhibitor (Roche, Dee Why, NSW, Australia) for 30 min on ice, then centrifugated 5 min at 13 000 r.p.m. For 2D gel electrophoresis, cell extracts were processed using 2D clean-up Kit (GE Healthcare, Ryldamere, Australia). The resulting immuno-precipitates were redissolved in 2D sample buffer (7 M urea, 2 M thiourea, 2% ASB-14, 1% DTT, 1% ampholytes), loaded onto 13 cm pI 4–7 IPG strips by passive re-hydration for 12 h and isoelectric focussing performed using a fast voltage gradient (8000 V max, 24 000 V/h) at 15 μC, using an Ettan IPGphor 3 system (GE Healthcare). Fractionation according to protein molecular weight (the second dimension) was carried out on 4–12% polyacrylamide gels using 4-12% Bis-Tris precast 8 × 13 cm gels (Novex NuPAGE, Invitrogen) at 75 V. 2D gels were electrophoretically transferred onto nitrocellulose filters using the iBlot dry blotting system, following the manufacturer's instructions (Invitrogen, Grand Island, NY, USA). Filters from both 1D and 2D western blots were probed with specific antibodies. Antibody binding was detected by incubation with goat anti-mouse, anti-rat or anti-rabbit IgG antibodies conjugated to HRP (SouthernBiotech, Birmingham, AL, USA) and chemiluminescence (ECL, GE Healthcare). Immunoprecipitation was performed using a rat monoclonal antibody to Bim (3C5, Enzo Life Sciences,[25] New York, NY, USA) and analyzed by western blotting.

Reagents and antibodies for western blot analysis. The MEK1/2 inhibitor U0126 (no. 9903), anti-Erk1/2 (no. 9102) and anti-phospho-Erk1/2 antibodies (no. 9101) were obtained from Cell Signaling (Danvers, MA, USA). Anti-Bcl-2 (no. 610538), anti-Bcl-x_L (no. 610212) and anti-Bax (no. 554106) antibodies were purchased from BD Biosciences (San Diego, CA, USA). Anti-Bim (no. ADI-AAP-330E) and anti-Bmf (clone 17A9) antibodies were from Enzo Life Sciences and anti-β-actin (AC-40) from Sigma-Aldrich (St. Louis, MO, USA). Anti-Bak (Ab-2) and anti-Mcl-1 (600-401-394) antibodies were from Oncogene Research (La Jolla, CA, USA) and Rockland (Gilbertsville, PA, USA), respectively.

T- and B-lymphocyte purification and mitogenic activation *in vitro*. T lymphocytes were purified from lymph nodes and spleens by depletion of all other cell types as described previously.[25] Unbound purified T lymphocytes (2×10^6 cells/ml) were cultured in DME medium supplemented with 10% (v/v) FBS, 250 mM L-asparagine (FMA) and T cells were activated with 2 ng/ml PMA plus 0.1 μg/ml ionomycin (both from Sigma-Aldrich) or with plate-bound hamster mAbs to CD3 (145-2C11) and CD28 (37N51), both at 10 μg/ml in the coating solution (PBS) plus saturating concentration of recombinant mouse IL-2.

B lymphocytes were purified from spleen using the $CD19^+$ B-cell isolation kit (Miltenyi Biotec, North Ryde, NSW, Australia). Such purified B cells (2×10^6 cells/ml) were cultured in FMA medium and were activated with 10 μg/ml F(ab')2 goat anti-mouse IgM Ab fragments (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and anti-CD40 (FGK45) monoclonal antibodies plus saturating concentrations of recombinant mouse IL-2, IL-4 and IL-5.

Cell purity (at least over 90% for each experiment) was verified by staining enriched T- or B-cell populations with specific antibodies to CD3 (145-2C11), CD4 (YTS169), CD8 (T24.3.21) or CD45R-B220 (5.1), IgM (11-26C), IgD (RB6-8C5), respectively, followed by FACS analysis. At 24, 48 and 120 h after treatment commenced, cells were stained with 2 μg/ml propidium iodide (PI) and anti-CD25 (PC-61) monoclonal antibody. Live and activated cells were considered as PI^- and $CD25^+PI^-$, respectively.

FACS analysis, cell surface and intracellular immunofluorescent staining. Cells for FACS analysis were stained with various fluorochrome-conjugated monoclonal antibodies for at least 20 min at 4 °C. Non-specific binding of antibodies to Fc-receptors was inhibited by adding 40% (v/v) supernatant of the anti-Fcγ-receptor monoclonal antibody producing hybridoma (24G2). Cells were then washed in FACS buffer (KDS BSS: 7.4 mM Hepes pH 7.2, 149 mM NaCl, 3.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.8 mM K_2HPO_4, 2% v/v FBS, 0.01 M NaN_3, 2 mM EDTA). For intracellular staining, thymocytes were fixed and stained using the BD Cytofix/Cytoperm fixation and permeabilisation kit, according to the manufacturer's instructions (BD Biosciences) with a rat anti-Bim mAbs (3C5 at 5 μg/ml; Enzo Life Science[25]) and analyzed by flow cytometry. Stained cells were analyzed on a FACScan (Beckton Dickinson, San Jose, CA, USA) and staining with propidium iodide was included in the final wash step to label dead cells.

Cell survival assays. $CD4^+CD8^+$ (DP) thymocytes were sorted as described previously[5] and incubated in FMA medium alone (cytokine withdrawal) or treated with ionomycin (up to 1 μg/ml), 10 ng/ml PMA, dexamethasone (up to 10 nM), etoposide (up to10 μg/ml), taxol (1 μg/ml) or ABT-737 (1 μM). Cell survival was quantified daily by staining with 2 μg/ml PI followed by FACS analysis. Specific death was calculated using the following equation: ((% apoptosis–% spontaneous apoptosis)/(100–% spontaneous apoptosis)).

Statistical analysis. Statistical analysis was performed using Student's *t* test. *P*-values less than 0.05 were considered as significant.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)