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Individual and overlapping roles of BH3-only proteins Bim and Bad in apoptosis of lymphocytes and platelets and in suppression of thymic lymphoma development

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BH3-only proteins, such as Bim and Bad, contribute to tissue homeostasis by initiating apoptosis in a cell type- and stimulus-specific manner. Loss of Bim provokes lymphocyte accumulation *in vivo* and renders lymphocytes more resistant to diverse apoptotic stimuli and Bad has been implicated in the apoptosis of haematopoietic cells upon cytokine deprivation. To investigate whether their biological roles in apoptosis overlap, we generated mice lacking both Bim and Bad and compared their haematopoietic phenotype with that of the single-knockout and wild-type (wt) animals. Unexpectedly, *bad*$^{-/-}$ mice had excess platelets due to prolonged platelet life-span. The *bim*$^{-/-}$*bad*$^{-/-}$ mice were anatomically normal and fertile. Their haematopoietic phenotype resembled that of *bim*$^{-/-}$ mice but lymphocytes were slightly more elevated in their lymph nodes. Although resting B and T lymphocytes from *bim*$^{-/-}$*bad*$^{-/-}$ and *bim*$^{-/-}$ animals displayed similar resistance to diverse apoptotic stimuli, mitogen activated *bim*$^{-/-}$*bad*$^{-/-}$ B cells were more refractory to cytokine deprivation. Moreover, combined loss of Bim and Bad-enhanced survival of thymocytes after DNA damage and accelerated development of γ-irradiation-induced thymic lymphoma. Unexpectedly, their cooperation in the thymus depended upon thymocyte–stromal interaction. Collectively, these results show that Bim and Bad can cooperate in the apoptosis of thymocytes and activated B lymphocytes and in the suppression of thymic lymphoma development.
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Programmed cell death (apoptosis), a genetically controlled process for eliminating unwanted cells, is essential for the normal development and function of multi-cellular organisms.[1] The Bcl-2 protein family has a major role in the regulation of apoptosis in vertebrates.[2] Its BH3-only members, which include Bad, Bim/Bod, Bik/Blk/Nbk, Hrk/DP5, Puma/Bbc3, Noxa, Bmf and Bid, are essential initiators of apoptosis that are activated in response to distinct developmental cues and cytotoxic signals.[3,4] They selectively bind to pro-survival members of the Bcl-2 family (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w and A1) and trigger mitochondrial release of cytochrome *c* by a mechanism requiring their pro-apoptotic relatives Bax or Bak.[5,6]

The BH3-only protein Bad was the first pro-apoptotic Bcl-2 family member found to be regulated by extracellular survival factors.[7] In cells stimulated with cytokines, such as IL-3, Bad is phosphorylated by AKT (and certain other kinases) on several serine residues, and this inhibits its pro-apoptotic activity by allowing its sequestration by 14-3-3 scaffold proteins or by directly preventing its interaction with Bcl-x_L or Bcl-2.[7,8] Conversely, in the absence of survival signals, Bad is de-phosphorylated, increasing its pro-apoptotic activity.[8,9] Perhaps surprisingly, however, *bad*$^{-/-}$ mice were found to be largely normal. Only minor defects in apoptosis were observed upon withdrawal of IGF-1, EGF or glucose in embryonic fibroblasts or mammary epithelial cells,[10,11] and some aged *bad*$^{-/-}$ mice developed lymphoma.[10] As Bad loss did not enhance the survival of cytokine-deprived myeloid progenitor cells,[12] its physiological role in growth factor withdrawal-induced apoptosis remains uncertain.

In contrast, the BH3-only protein Bim, which is regulated by a range of transcriptional and post-translational mechanisms,[13] have a major role in the death of haematopoietic cells induced by cytokine deprivation and certain other apoptotic stimuli, including deregulated calcium flux and, although to a lesser extent, DNA damage.[14–16] Within the animal, Bim loss provokes accumulation of haematopoietic cells, particularly lymphocytes impairs deletion of autoreactive thymocytes[17] and B cells,[18] and is required for shut-down of T cell immune responses.[19–21] Furthermore, in certain circumstances, Bim deficiency can provoke autoimmune disease[14] and contribute to lymphoma development.[22]

Despite its major role in haematopoietic cell homeostasis and cytokine deprivation-induced apoptosis,[14] the loss of Bim provokes less marked effects than the combined loss of Bax and Bak[23,24] or Bcl-2 overexpression,[15,25] suggesting

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Abbreviations: DNA, deoxyribonucleic-acid; EGF, epidermal growth factor; ERK, Extracellular signal-regulated kinases; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; IGF, insulin-like growth factor; IL, interleukin; PI3K, Phosphoinositide 3-kinase; SD, standard deviation; SEM, standard error of the mean; UV, ultra-violet
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that Bim has overlapping functions with other BH3-only proteins. Indeed, although mice lacking Bik are essentially normal,[26] loss of both Bik and Bim arrests spermatogenesis,[27] whereas combined loss of Puma and Bim renders haematopoietic cells more refractory to diverse apoptotic stimuli, including cytokine deprivation, than loss of either alone.[16]

As Bad and Bim have been implicated in the apoptosis signalling driven by cytokine withdrawal and DNA damage, as well as in tumorigenesis, we have investigated their potential overlapping functions in cell death by generating Bim/Bad double knockout mice. By analysis of their phenotype, and of purified lymphoid populations in culture, we show that Bim and Bad cooperate in certain apoptotic responses and in the suppression of γ-irradiation-induced thymic lymphoma. Unexpectedly, some of their cooperation *in vivo* appears to involve lymphocyte–stromal cell interactions.

Results

Lymphoid hyperplasia in Bim-deficient mice is slightly increased by concomitant loss of Bad. All progeny of $bim^{+/-}bad^{+/-}$ and $bim^{+/+}bad^{-/-}$ intercrosses developed in the expected Mendelian ratios, but intercrosses of $bim^{+/-}bad^{-/-}$ animals yielded less than the expected number of $bim^{-/-}bad^{-/-}$ double-knockout offspring (Supplementary Table S1). Their deficit, however, was similar to that of $bim^{-/-}$ progeny from $bim^{+/-}$ intercrosses ([14] and P Bouillet unpublished observations). Thus, the loss of Bad does not augment the penetrance of embryonic death evoked by Bim deficiency.

Bim/Bad doubly deficient mice were fertile and their body and organ weights (Supplementary Figure S1), appearance and behaviour were normal. As Bim[28] and Bad[29] are both expressed in lymphoid and myeloid cells, we analysed the haematopoietic compartment of 6 to 12-week-old $bim^{-/-}bad^{-/-}$ and control (wt, $bim^{-/-}$, $bad^{-/-}$) mice to determine whether their combined loss exacerbated the leukocyte accumulation in $bim^{-/-}$ mice.[14] Consistent with previous studies,[10,14] $bad^{-/-}$ mice had normal numbers of lymphoid and myeloid cells, whereas Bim-deficient mice had elevated mature B and T cells (Figure 1). The $bim^{-/-}bad^{-/-}$ mice displayed increases in B and T lymphocytes similar to those of $bim^{-/-}$ mice (Figure 1). However, the lymph node cellularity in mice lacking both Bim and Bad was slightly albeit significantly higher ($P=0.05$) than in $bim^{-/-}$ mice. There was a trend towards increased numbers of granulocytes in spleens of $bim^{-/-}bad^{-/-}$ mice (Figure 1) but this did not reach statistical significance. In addition, we found (significantly) increased numbers of nucleated erythroid cells in spleens of $bim^{-/-}bad^{-/-}$ and to a similar extent also in $bim^{-/-}$ mice (Figure 1). We believe, however, that this is not due to abnormally increased survival of these cells but a consequence of a shift of erythropoiesis from the marrow to the spleen (extra-medullary erythropoiesis) due to the overcrowding of the bone marrow by supernumerary B lymphoid cells, as also seen in $E\mu$-*bcl-2* transgenic mice.[30]

Since loss of Bim elevates serum immunoglobulin (Ig) levels,[14] we measured the levels of IgG and IgM by ELISA in the sera of mice either 6–12 weeks or >20-week-old. Whereas loss of Bim provoked a ~5-fold increase in total serum IgG and IgM levels over wt mice, naïve $bad^{-/-}$ mice

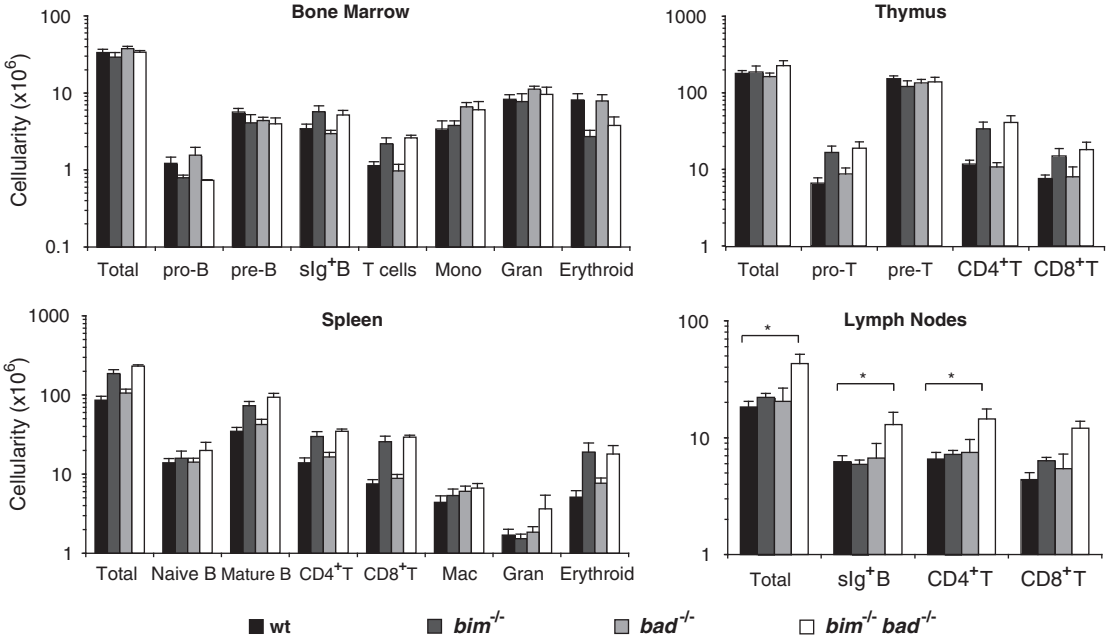

Figure 1 Haematopoietic cell subset composition of $bim^{-/-}bad^{-/-}$ and control mice. Cell subset composition analysis of bone marrow, thymus, spleen and lymph nodes (pooled axillary, inguinal and mesenteric) from 6 to 12-week-old $bim^{-/-}bad^{-/-}$ and control $bim^{-/-}$, $bad^{-/-}$ or wt mice. Single cell suspensions were stained with fluorochrome-conjugated surface marker-specific monoclonal antibodies, and the percentages in each cellular compartment quantified by flow cytometric analysis. Total cell numbers were determined by trypan blue exclusion and counting in a haemocytometer. Data represent mean absolute cell numbers ± S.E. of 4–7 mice of each genotype from at least four independent experiments. An asterisk denotes $P<0.05$ significance in differences between the indicated populations

showed no increase, and loss of Bim plus Bad did not elevate the levels over loss of Bim alone (Supplementary Figure S2).

Bim-deficient animals (on a mixed C57BL/6x129SV background) develop an autoimmune disorder resembling human systemic lupus erythematosus,[14] whereas mice lacking Bad were reported to develop diffuse large B cell lymphoma late in life.[10] Hence, we monitored cohorts of $bim^{-/-}bad^{-/-}$, $bim^{-/-}$, $bad^{-/-}$ and wt animals, all on a C57BL/6 background (8–20 generations backcrossed), until 500 days of age for signs of autoimmune disease or haematological malignancy. Only a single $bim^{-/-}bad^{-/-}$ mouse succumbed to glomerulonephritis, and none of the 18 other $bim^{-/-}bad^{-/-}$ animals or any of the $bad^{-/-}$ ($n=21$) or $bim^{-/-}$ ($n=20$) aged mice examined developed any disease. Hence, the autoimmune disorder that previously manifested in mice lacking Bim appears to depend upon genetic background, even when Bad is also absent, and genetic background may well also have contributed to the low-penetrance lymphoid malignancy reported for aged Bad-deficient mice.[10]

Bad deficiency increases platelet numbers and life-span. The Bcl-2 family governs the circulating life-span of platelets. These anuclear cells depend on Bcl-x_L for survival, which functions to restrain pro-apoptotic Bak. Mutations in Bak extend platelet life span and cause thrombocytosis.[31] We unexpectedly found that Bad-deficient mice had elevated platelet numbers in their peripheral blood (Figure 2a), revealing a previously unidentified role for Bad in platelet homeostasis. Western blotting showed that platelets contain Bad protein (Figure 2b), and since the bone marrow and spleen of $bad^{-/-}$ mice showed normal numbers of megakaryocytes (data not shown), we hypothesized that platelet life-span, rather than production, was perturbed. We therefore examined platelet clearance by labelling platelets with biotin and tracking their survival *in vivo*. Platelet half-life — defined as the period during which 50% of labelled platelets had disappeared from circulation — was significantly (albeit modestly) increased in $bad^{-/-}$ mice (Figure 2c). To examine whether this effect was cell-intrinsic, we performed adoptive transfers of labelled platelets. Upon transfer into wt recipients, the rate of disappearance of $bad^{-/-}$ platelets was decreased compared with wt platelets (Figure 2d). Thus, Bad regulates platelet life-span *in vivo* in a cell-intrinsic manner.

The increased platelet numbers in $bad^{-/-}$ mice contrasts with the mild thrombocytopenia previously reported for Bim-deficient animals,[14] which appears to be due to a reduction in platelet production by megakaryocytes. Remarkably, mice lacking both Bim and Bad had platelet numbers comparable with wt animals (Figure 2a), presumably because the reduced platelet production (conferred by Bim-deficiency) was counter-balanced by the enhanced platelet survival (resulting from loss of Bad).

Sensitivity of $bim^{-/-}bad^{-/-}$ lymphocytes to apoptotic stimuli *in vitro*. Bim is critical of the apoptosis of lymphocytes evoked by diverse cytotoxic insults, including

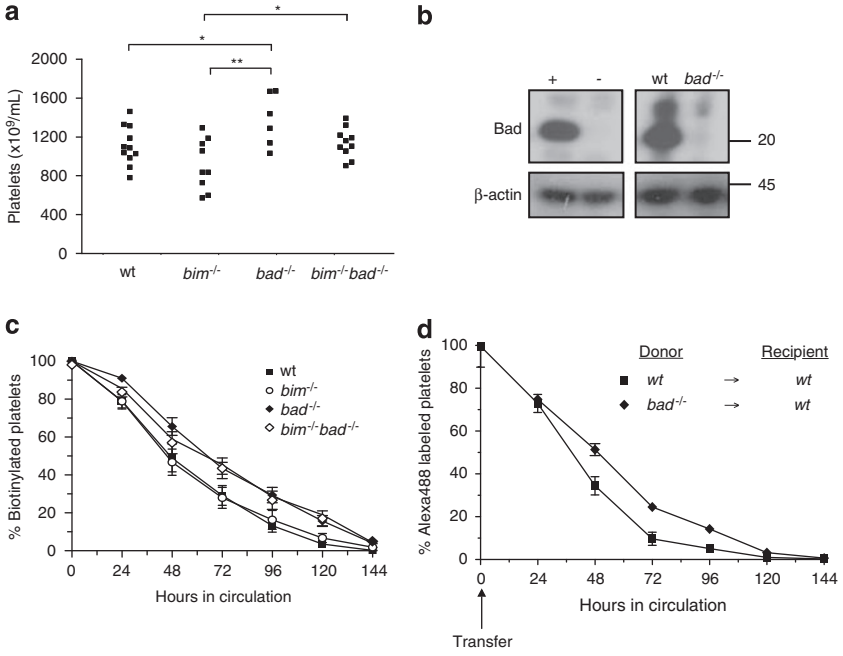

Figure 2 $bad^{-/-}$ but not $bim^{-/-}bad^{-/-}$ mice have abnormally increased numbers of platelets. (**a**) Peripheral blood platelet numbers were determined from 6- to 12-week-old $bim^{-/-}bad^{-/-}$, $bim^{-/-}$, $bad^{-/-}$ or wt mice by automated counting (Advia 2120, Bayer). Data represent absolute numbers ($\times 10^9$/ml) of 6–12 mice of each genotype. *$P<0.05$. (**b**) Western blot showing expression of Bad in wt platelet lysates and its absence in platelets derived from $bad^{-/-}$ mice. The positive control (+) for Bad expression was a lysate of wt mouse embryonic fibroblasts and the negative control (−) a lysate of $bad^{-/-}$ mouse embryonic fibroblasts. Results are representative of two independent experiments. (**c**) Platelet half-life in the circulation was determined in 6 to 12-week-old $bim^{-/-}bad^{-/-}$ ($n=6$), $bim^{-/-}$ ($n=10$), $bad^{-/-}$ ($n=8$) or wt ($n=16$) mice by injection of biotin and flow cytometric analysis of the disappearance of $CD41^+biotin^+$ platelets (see Materials and methods). Data represent mean ± S.D. (**d**) The half-life of transplanted Bad-deficient platelets in the circulation of 8-week-old wt recipient mice ($n=8$) was determined by adoptive transfer. Alexa488-labelled platelets, derived from $bad^{-/-}$ ($n=4$) or wt ($n=4$) donor mice, were injected into wt recipients and the disappearance of $CD41^+Alexa488^+$ platelets monitored by flow cytometry (see Materials and methods). Data represent mean ± S.D.

cytokine withdrawal and deregulated calcium flux,[14] and contributes modestly to DNA damage-induced apoptosis,[15] even though the *bim* gene lacks a binding site for the tumour suppressor p53.[32] To determine whether Bim and Bad have overlapping roles in apoptosis induction, we purified lymphoid sub-populations from $bim^{-/-}bad^{-/-}$ and control (wt, $bim^{-/-}$, $bad^{-/-}$) mice and monitored their survival after exposure to a range of apoptotic stimuli *in vitro*. Bad-deficient lymphocytes were normally sensitive to cytokine deprivation and to DNA damage, evoked either by etoposide or γ-irradiation (Figure 3). As reported,[14,15] $bim^{-/-}$ thymocytes were considerably more resistant than their wt counterparts to cytokine withdrawal and showed minor, albeit significant resistance to γ-irradiation and etoposide (Figure 3). However, combined loss of Bim and Bad did not enhance thymocyte survival *in vitro* more than loss of Bim alone (Figure 3).

Bim-deficient mature B and T lymphocytes ($CD4^+8^-$ as well as $CD4^-8^+$) were, as reported,[14] significantly protected against diverse cytotoxic stimuli, including cytokine deprivation and exposure to dexamethasone, etoposide or γ-irradiation. However, the concomitant absence of Bad did not provide extra protection (Figure 3, Supplementary Figure S3 and data not shown).

Because the signalling pathways that regulate apoptosis often differ between quiescent and activated cells, we mitogenically stimulated B and T cells from $bim^{-/-}bad^{-/-}$ and control mice and monitored their survival *in vitro*. As reported,[14] ConA-activated T lymphoblasts from $bim^{-/-}$ mice were refractory to IL-2 deprivation and modestly protected against γ-irradiation, but the $bim^{-/-}bad^{-/-}$ T lymphoblasts died at the same rate as their $bim^{-/-}$ counterparts (Figure 3 and data not shown). We also examined splenic B lymphocytes activated *in vitro* with lipopolysaccharide plus IL-2, IL-4 and IL-5. Following cytokine deprivation, the survival of $bad^{-/-}$ and wt B cell blasts was similar, whereas $bim^{-/-}$ B lymphoblasts were highly protected (Figure 3). Interestingly, activated B cells from $bim^{-/-}bad^{-/-}$ mice were modestly, but significantly ($P<0.05$), more refractory to cytokine deprivation than $bim^{-/-}$ cells (Figure 3). Thus, Bim and Bad have an overlapping role in the control of apoptosis in activated B lymphocytes.

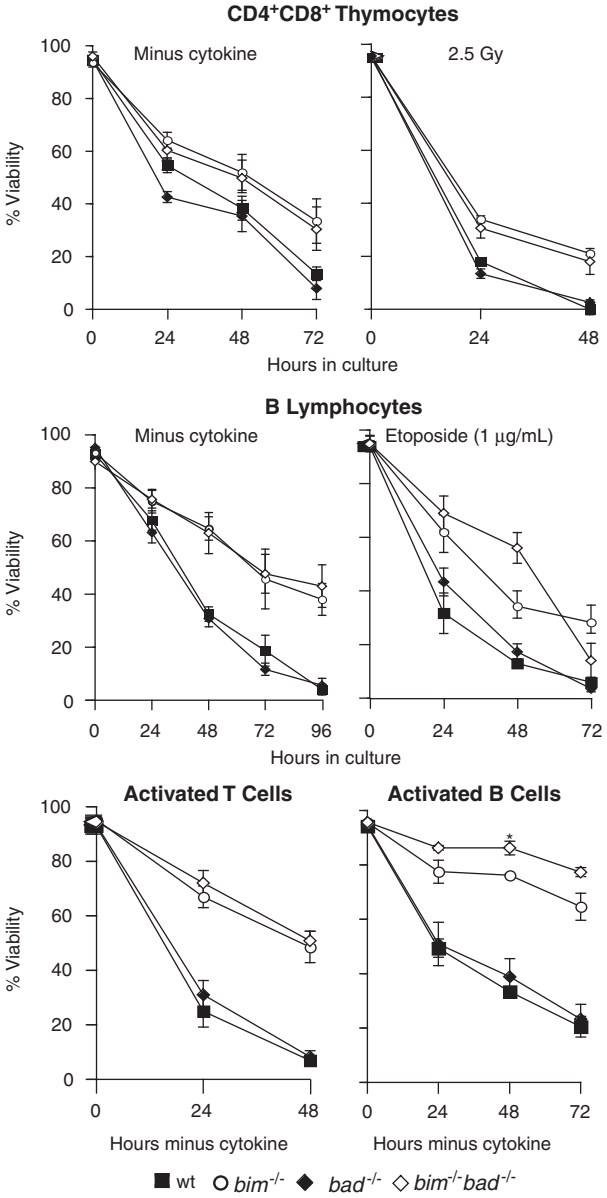

Figure 3 Susceptibility of $bim^{-/-}bad^{-/-}$ lymphocytes to apoptotic stimuli in culture. $CD4^+8^+$ thymocytes and mature B cells ($B220^+sIgM^{lo}sIgD^{hi}$) were FACS sorted from thymus or lymph node cell suspensions, respectively, from 6- to 12-week-old $bim^{-/-}bad^{-/-}$, $bim^{-/-}$, $bad^{-/-}$ or wt mice and cultured with etoposide (1 μg/ml) or subjected to cytokine withdrawal or γ-irradiation (2.5 Gy). The activated T or B cell blasts were cultured in the absence of cytokines for the indicated times and then stained with FITC-coupled Annexin V plus propidium iodide and the percentages of viable cells quantified by FACS. Data represent mean ± S.D. of cells from 3 to 5 mice of each genotype

Bim and Bad both contribute to γ-irradiation-induced thymocyte apoptosis *in vivo*. DNA damage potently elicits thymocyte apoptosis, mediated largely,[33,34] albeit not exclusively,[35] via the tumour suppressor p53. In this response, Puma, a direct p53 transcriptional target, is the rate-limiting BH3-only protein,[15,36–38] but Bim also contributes[14,15] and Bad might also have a role.[10,39,40]

To explore the roles of Bim and Bad in the DNA damage response, we subjected wt and p53-deficient mice to γ-irradiation (5 Gy), harvested their thymi 0, 4, 8 and 12 h later, and assessed Bim and Bad expression by Western blotting. Bim levels increased in a p53-independent manner: as early as 4 h after γ-irradiation, Bim was detectable at similar levels in both wt and $p53^{-/-}$ thymocytes (Figure 4a). By comparison, although the available antibody readily detected Bad in wt fibroblasts (Figure 4a, lane 5), Bad levels were low in untreated wt as well as $p53^{-/-}$ thymocytes and did not noticeably increase after γ-irradiation (Figure 4a).

Next, we examined the impact of loss of Bim, Bad or both on the response of lymphoid cells to DNA damage *in vivo*. Animals were exposed to 5 Gy γ-irradiation and their thymus, lymph nodes and spleen harvested 20 h later. Cell numbers in each haematopoietic organ were determined and the cell subset composition quantified by immunofluorescent staining with surface marker-specific antibodies and FACS analysis. As reported,[41] wt $CD4^+8^+$ thymocytes were highly sensitive

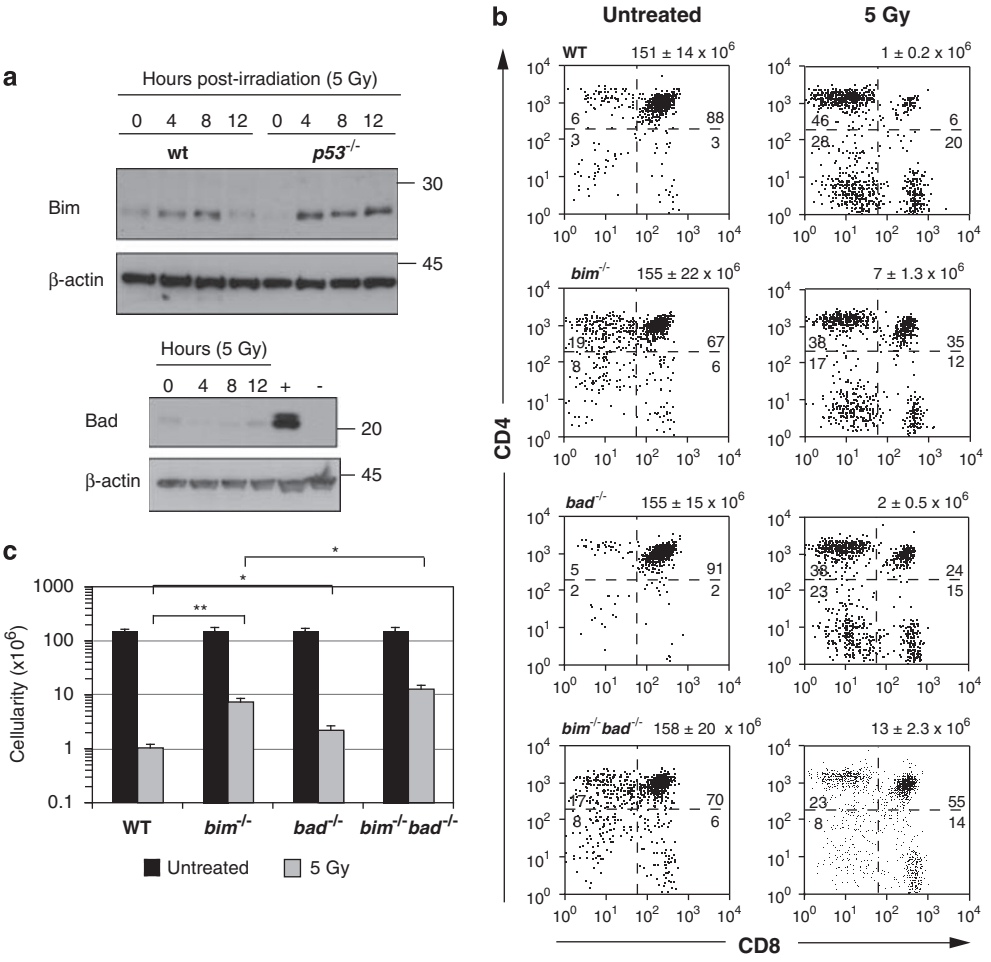

Figure 4 Bim and Bad contribute to γ-irradiation-induced thymocyte apoptosis *in vivo*. (**a**) Western blot showing the effect of γ-irradiation on Bim and Bad expression in thymocytes, with β-actin as a loading control. Wt or p53-deficient mice were left untreated or exposed to γ-irradiation (5 Gy) and lysates made of thymi harvested 0, 4, 8 and 12 h later. The positive control (+) for Bad expression was a lysate of wt mouse embryonic fibroblasts and the negative control (−) a lysate of *bad*$^{-/-}$ thymocytes. Results are representative of two independent experiments. (**b**) Effect of γ-irradiation on thymic cell composition. Wt, *bim*$^{-/-}$, *bad*$^{-/-}$ or *bim*$^{-/-}$*bad*$^{-/-}$ mice were exposed to γ-irradiation (5 Gy). Thymi were harvested 20 h later, and cell viability determined by counting single cell suspensions in a haemocytometer. Cells were stained with monoclonal antibodies to CD4 and CD8 to determine the indicated percentages of the four major thymocyte subsets. The numbers of CD4$^+$8$^+$ thymocytes ± S.D. before and after γ-irradiation are shown above the panels. (**c**) The number of CD4$^+$8$^+$ thymocytes following γ-irradiation. Data represent the mean ± S.D. from three independent experiments. $P<0.05$ (*) and $P<0.01$ (**) are indicated

to γ-irradiation (Figure 4b), their number plummeting by ~150-fold (Figure 4c). The Bad-deficient CD4$^-$8$^-$ (pro-T) as well as mature CD4$^+$8$^-$ and CD4$^-$8$^+$ thymocytes were killed as efficiently as their wt counterparts. Surprisingly, however, the percentages and total numbers of surviving CD4$^+$8$^+$ thymocytes were ~2-fold higher ($P<0.05$) in *bad*$^{-/-}$ than wt mice (Figure 4b). As reported,[15] *bim*$^{-/-}$ CD4$^+$8$^+$ thymocytes were markedly more resistant than wt cells ($P<0.0001$) to γ-irradiation *in vivo* (Figure 4b and c). Notably, the combined loss of Bad plus Bim allowed greater survival ($P<0.05$) than loss of either BH3-only protein alone (Figure 4b and c). Unlike the CD4$^+$8$^+$ thymocytes, however, the mature CD4$^+$ or CD8$^+$ T cells and B cells in *bad*$^{-/-}$*bim*$^{-/-}$ mice responded to γ-irradiation similarly to their *bim*$^{-/-}$ counterparts, and loss of Bad alone was not protective (Supplementary Figure S4). These results show that Bim and Bad have overlapping functions in γ-irradiation-induced killing of CD4$^+$8$^+$ thymocytes within the animal but that Bad does not contribute significantly to the death of their mature B or T cells.

Bad contributes to γ-irradiation-induced apoptosis of CD4$^+$8$^+$ thymocytes through a haematopoietic cell-extrinsic mechanism. The increased survival of CD4$^+$8$^+$ thymocytes in γ-irradiated *bad*$^{-/-}$ mice (Figure 4b and c) was surprising because the expression of Bad in thymocytes was exceedingly low and did not increase after DNA damage (Figure 4a). Thymocyte survival is influenced by its microenvironment, a network of epithelial and haematopoietic cells, including dendritic cells and macrophages.[42] The observations that *bad*$^{-/-}$ CD4$^+$8$^+$ thymocytes showed enhanced survival to γ-irradiation within the animal (Figure 4b and c) but not as purified cells in culture (Figure 3) suggested that Bad might promote apoptosis of

$CD4^+8^+$ thymocytes indirectly through a thymocyte-extrinsic mechanism.

To explore this hypothesis, we generated sets of chimaeric animals by reconstituting lethally irradiated wt or *bad*$^{-/-}$ recipient mice with either a wt or *bad*$^{-/-}$ haematopoietic system. At 8–12 weeks post-reconstitution, the mice were γ-irradiated (5 Gy) and the surviving $CD4^+8^+$ thymocytes enumerated 20 h later. Both the wt as well as *bad*$^{-/-}$ thymocytes developing within a Bad-deficient stroma survived γ-irradiation significantly better ($P<0.05$) than wt or *bad*$^{-/-}$ thymocytes in a wt environment (Figure 5). The ~2-fold enhanced survival of wt thymocytes in *bad*$^{-/-}$ recipients was comparable with that of *bad*$^{-/-}$ thymocytes recovered from *bad*$^{-/-}$ recipients, and to that of thymocytes within un-manipulated (that is, non-chimaeric) *bad*$^{-/-}$ animals (compare Figures 4 and 5). These results show, surprisingly, that Bad must contribute to γ-irradiation-induced apoptosis of $CD4^+8^+$ thymocytes within the animal by acting in non-haematopoietic cells, most likely thymic epithelial cells (see Discussion). Pertinently, γ-irradiation provokes both transient and persistent changes to the cellular microenvironment that can facilitate tumorigenesis.[43]

Combined loss of Bim and Bad accelerates γ-irradiation-induced thymic lymphoma development. The reproducible development of thymic lymphoma by C57BL/6 mice subjected to fractionated low doses of γ-irradiation[44] provides a robust experimental test for candidate tumour suppressors. Bim[22,45,46] and to a lesser extent Bad[10] have been implicated as tumour suppressors. As both contribute to DNA damage-induced apoptosis of thymocytes, albeit Bim in a haematopoietic cell intrinsic (Figure 4) and Bad in an extrinsic manner (Figure 5), we investigated how their individual or combined loss affected γ-irradiation-induced thymic lymphoma development. We exposed cohorts of wt ($n=10$), *bim*$^{-/-}$($n=16$), *bad*$^{-/-}$ ($n=22$), *bim*$^{-/-}$*bad*$^{-/-}$ ($n=10$) and, as a control, *p53*$^{-/-}$ ($n=5$) mice to fractionated low dose (4 weekly doses of 1.5 Gy) γ-irradiation[44] and monitored them daily for signs of malignancy.

As reported,[47] thymic lymphoma developed considerably faster and with higher incidence in *p53*$^{-/-}$ mice (50% mortality by 14 weeks and 100% mortality by 18 weeks after the final dose of γ-irradiation) than in wt animals (10% by 14 weeks and ~50% by 57 weeks; Kaplan–Meier log rank $P<0.001$). Although some old *bad*$^{-/-}$ mice were reported to develop B lymphoma,[10] their development of thymic lymphoma (~50% incidence, median latency 60 weeks) was comparable with that of wt mice (Figure 6). Although Bim deficiency (even loss of one allele) markedly accelerates B lymphoma development in Eμ-*myc* transgenic mice,[22] *bim*$^{-/-}$ and wt mice developed γ-irradiation-induced thymic lymphoma with similar onset and incidence. Remarkably, however, the combined loss of Bim and Bad substantially accelerated ($P<0.04$) thymic lymphomagenesis (~80% incidence, median latency 23 weeks) over that in wt animals (~50% incidence, median latency 60 weeks; Figure 6). Histological evaluation of thymic sections from mice of all genotypes revealed a similar extent of tumour dissemination and disruption of the thymic architecture by infiltration of large blast cells (data not shown). Moreover, immuno-phenotyping revealed that most thymic lymphomas, regardless of genotype, were Thy-$1^+CD4^+CD8^+$ and displayed CD43 and/or CD44 (data not shown). All were transplantable into histocompatible recipients, producing large tumours within 40 days. These results show that Bim and Bad have overlapping roles as tumour suppressors, at least in this model of γ-irradiation-induced thymic lymphomagenesis.

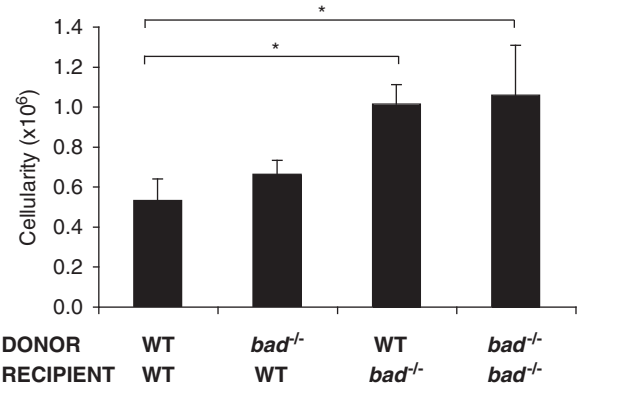

Figure 5 Bad contributes to γ-irradiation-induced thymocyte apoptosis in a haematopoietic cell-extrinsic manner. Four combinations of bone marrow-derived chimaeric mice were generated by adoptively transferring bone marrow cells from wt or *bad*$^{-/-}$ mice into lethally irradiated (2×5.5 Gy, 3 h apart) wt or *bad*$^{-/-}$ recipients. The reconstituted animals were exposed to 5 Gy whole body γ-irradiation 8–12 weeks post-reconstitution, and the percentages and total numbers of surviving $CD4^+8^+$ thymocytes determined 20 h later as described in Figure 4. Data represent mean ± S.D. from three independent experiments from 4–8 mice of each chimaeric type. Asterisks indicate statistically significant differences, $P<0.05$

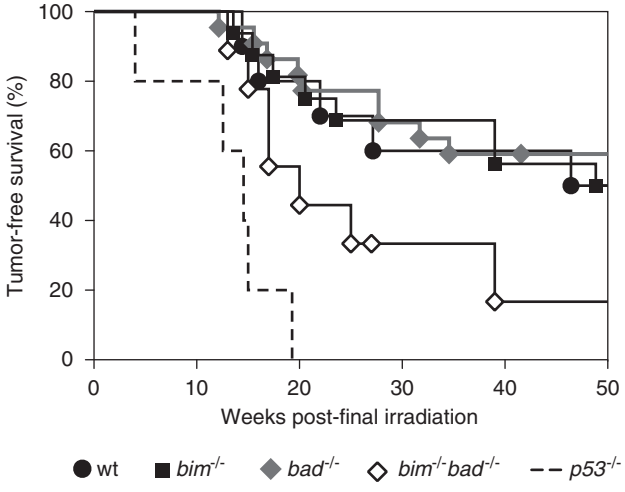

Figure 6 Concomitant loss of Bim and Bad accelerates γ-irradiation-induced thymic lymphoma development. Kaplan–Meier representation of tumour latency in *bim*$^{-/-}$*bad*$^{-/-}$ and control *bim*$^{-/-}$, *bad*$^{-/-}$, wt and *p53*$^{-/-}$ mice following four weekly doses of 1.5 Gy whole body γ-irradiation. *bim*$^{-/-}$*bad*$^{-/-}$ mice developed γ-irradiation-induced thymic lymphoma significantly faster ($P<0.04$) than control mice. Mice were monitored daily for signs of tumour development (hunched posture, laboured breathing and increased spleen/lymph nodes) and sick mice were killed and tissues analysed immediately

Discussion

Since both Bim[48,49] and Bad[7,8,50] can be activated by growth factor deprivation and certain other apoptotic stimuli, we generated Bim/Bad double knockout mice to investigate potential overlaps in their apoptotic function, both within the animal and in cultured lymphoid populations. Perhaps surprisingly, the *bim*$^{-/-}$*bad*$^{-/-}$ mice showed only slightly greater lymphadenopathy than the *bim*$^{-/-}$ animals (Figure 1), and their resting lymphocytes were no more resistant to cytokine deprivation than the Bim-deficient cells (Figure 3 and Supplementary Figure S3). The absence of synergy between Bim and Bad in these cells is compatible with a model in which neutralisation of all Bcl-2-like pro-survival proteins expressed within a given cell is required to initiate apoptosis efficiently.[51–53] Because Bcl-2 and Mcl-1 are both highly expressed in resting mature B and T lymphocytes and critical for their survival,[54,55] complete neutralisation of both these proteins is probably required for their efficient killing. Although Bim and Puma are particularly potent killers because they can engage all the pro-survival proteins, Bad is much less effective because it cannot bind to Mcl-1 (or A1).[51,52] Thus, in any cell whose survival is sustained by Mcl-1, Bad may only marginally boost Bim's pro-apoptotic function. In accord with that view, loss of both Puma and Bim elicits more profound and widespread effects on the lymphoid compartment[16] than combined loss of Bim plus Bad.

Bim and Bad do not cooperate in the development of autoimmunity or spontaneous malignancy. Although quiescent *bim*$^{-/-}$*bad*$^{-/-}$ and *bim*$^{-/-}$ B lymphocytes showed similar resistance to cytokine deprivation *in vitro*, mitogen-activated B cells from the doubly-deficient animals were more refractory than Bim-deficient B lymphoblasts (Figure 3). This difference may be because B lymphoblasts express higher levels than resting B cells of pro-survival proteins that Bad can neutralise, such as Bcl-x_L.[56] The modest increase in resistance of *bim*$^{-/-}$*bad*$^{-/-}$ B cell blasts to cytokine deprivation did not correlate, however, with notable evidence of an abnormally activated immune system. Although many *bim*$^{-/-}$*bad*$^{-/-}$ mice presented with larger lymph nodes than *bim*$^{-/-}$ mice, their numbers of mature B or T cells and their serum Ig levels were not significantly elevated. Moreover, although a fatal SLE-like autoimmunity was prevalent for *bim*$^{-/-}$ mice on a mixed C57BL/6x129SV genetic background,[14] the *bim*$^{-/-}$ and *bim*$^{-/-}$*bad*$^{-/-}$ mice analysed in this study, all extensively backcrossed to C57BL/6 animals, were not highly susceptible to autoimmune disease, so genetic background clearly has a critical role. Similarly, although it has been reported that aged ($\sim$18 month-old) *bad*$^{-/-}$ mice are prone to develop diffuse large B cell lymphoma,[10] we observed no malignancies in cohorts of *bad*$^{-/-}$ or even *bim*$^{-/-}$*bad*$^{-/-}$ animals monitored for a comparable period. Genetic background probably accounts for this difference, because the previous study involved mice on a mixed C57BL/6x129SV background. Pertinently, the 129SV background promotes tumorigenesis in other models of malignancy.[57]

Distinct roles for Bad and Bim in platelet homeostasis. We were intrigued to find that mice lacking Bad (but not those lacking Bim) have a modest elevation in peripheral blood platelet number (Figure 2a). Our data suggest that the basis for this effect is not an increase in their production, as megakaryocyte numbers were not elevated, but rather an extension of circulating platelet half-life (Figure 2c). Platelet life-span is known to be regulated by the Bcl-2 family of proteins, in particular pro-survival Bcl-x_L and pro-apoptotic Bak.[31] Platelets depend on Bcl-x_L to restrain Bak and maintain viability in the circulation.[31,58] Loss of function mutations in Bcl-x_L cause dose-dependent reductions in platelet life-span[31] and pharmacological inhibition of Bcl-x_L with the BH3-mimetic ABT-737[59] triggers platelet apoptosis and thrombocytopenia, apparently by freeing Bak and Bax from Bcl-x_L. The mechanism by which this occurs at steady state *in vivo* is still unclear. One possibility is that BH3-only proteins regulate the entry into apoptosis. Intriguingly, a recent study has suggested that Akt-mediated inactivation of Bad facilitates the survival of human platelets *in vitro*.[60] Our data show that in the absence of Bad, platelet life span is extended *in vivo*. Since ABT-737 has the same binding specificity as Bad (Bcl-2, Bcl-x_L and Bcl-w),[61] Bad probably contributes to the initiation of physiological platelet apoptosis by neutralising Bcl-x_L.

Bim and Bad contribute in distinct ways to γ-irradiation-induced apoptosis of thymocytes *in vivo*. The major regulator of the cellular response to DNA damage is the p53 tumour suppressor,[62] which appears to mediate apoptosis primarily by inducing Puma, with minor contributions from Noxa.[15,36–38] However, p53-independent DNA damage-induced apoptotic pathways exist, as illustrated by the apoptosis following γ- or UV-irradiation observed in many tumour cells lacking functional p53.[35] Indeed, Bim is upregulated by DNA damage in γ-irradiated p53-deficient thymocytes (Figure 4a), and loss of Bim can protect (albeit only to a minor extent) certain lymphoid cell types from DNA damage-induced apoptosis both *in vitro*[14] and *in vivo*.[15] How genotoxic insults upregulate Bim is uncertain, but a plausible intermediary is FOXO3A, as it is a critical inducer of *bim* transcription in cytokine-deprived haematopoietic cells[48] and can be activated by DNA damage in a p53-independent manner.[63]

Surprisingly, loss of Bad had no effect on γ-irradiation-induced thymocyte killing *in vitro* (Figure 3), but it diminished their death *in vivo* (Figure 4b and c). Analysis of chimaeric mice (Figure 5) showed that this protective effect was not intrinsic to the $CD4^+8^+$ thymocyte population but rather due to loss of Bad within the thymic microenvironment. These results suggest that following γ-irradiation a nurturing stromal cell population lacking Bad survives better, presumably rendering it more able to sustain the survival of the thymic T lymphoid cells.

Bim and Bad cooperate in the suppression of γ-irradiation-induced thymic lymphoma development. Using the classic low dose γ-radiation-induced thymic lymphoma model,[44] we found that combined loss of Bim and Bad, albeit not loss of either BH3-only protein alone, accelerated tumorigenesis, showing functional overlap between these BH3-only proteins in tumour suppression.

Loss of p53 was more potent in accelerating thymic lymphoma development than combined loss of Bim and Bad; this is consistent with the capacity of p53 to activate multiple tumour suppressive mechanisms in addition to apoptosis, including cell cycle arrest and senescence.[62]

Since Bim and Bad are not thought to be involved in p53-induced apoptosis, how might their combined loss accelerate γ-irradiation-induced thymic lymphoma development? It is notable that after γ-irradiation, thymocytes are considerably more numerous in *bim*$^{-/-}$ and *bim*$^{-/-}$*bad*$^{-/-}$ animals than in wt mice (Figure 4b). Thus, protection conferred by the absence of Bim and/or Bad substantially increases the size of the putative susceptible target population. Furthermore, the elimination of more than 90% of the more mature cells by the γ-irradiation (Figure 4b) will provide a strong feedback signal to the immature cells, including nascent neoplastic clones, to proliferate and regenerate the thymus. Indeed, the repeated γ-irradiation doses both re-enforce this impetus and provide the mutagenesis contributing towards malignant progression.[44] Our observation that Bad deficiency protects the thymic microenvironment from the irradiation (Figure 5) suggests that the stroma lacking Bad can more effectively sustain the emerging neoplastic clones. Accordingly, we propose that the accelerated lymphoma development in *bim*$^{-/-}$*bad*$^{-/-}$ animals may reflect a concomitant cell-autonomous enhancement of survival in immature thymocytes, mediated largely by the absence of Bim, and a haematopoietic-extrinsic contribution by a more robust stromal support population, promoted primarily by the absence of Bad. This interpretation of the data would coincide with the view that tumorigenesis often relies on changes in the microenvironment of the emerging malignant clone.[43] Alternatively, since bone marrow resident haematopoietic stem/progenitor cells that have sustained oncogenic lesions are critical for γ-radiation-induced thymic lymphoma development,[44] it is also possible that combined loss of Bim and Bad accelerates tumorigenesis by extending the survival of such 'lymphoma stem cells'.

Evidence is emerging that combined activation of Bim and Bad is not only important for tumour suppression but also has a critical role in anti-cancer therapy. The killing of Bcr-Abl-transformed cells by Imatinib (Gleevec) requires Bim and to a lesser extent Bad,[64] which are both activated by shutdown of PI3K/AKT and ERK signalling. Consequently, our results suggest that treatment of certain types of lymphomas might be improved by agents that target these signalling pathways.

Materials and Methods

Mice. All animal work followed the guidelines of the Melbourne Directorate Animal Ethics Committee. The generation and genotyping of *bim*$^{-/-}$ mice,[14] *bad*$^{-/-}$ mice[10] and *p53*$^{-/-}$ mice[65] has been described previously. These strains of mice were all originally generated on a mixed C57BL/6 $\times$ 129SV background, using 129SV-derived ES cells, but had been backcrossed with C57BL/6 mice for 8–15 generations before use in the studies described here. The *bim*$^{-/-}$*bad*$^{-/-}$ mice were generated by intercrossing *bim*$^{-/-}$ and *bad*$^{-/-}$ mice. Animals were analysed at 6–12 weeks of age unless otherwise specified.

Immunofluorescence staining, flow cytometric analysis and cell sorting. Single cell suspensions were prepared from bone marrow, spleen, lymph nodes, peripheral blood or thymus. Cell subset composition was determined by immunofluorescent staining with the following rat or hamster monoclonal antibodies: RA3-6B2 (anti-B220), S7 (anti-CD43), 333.12 or 5.1 (anti-IgM), 11-26C (anti-IgD), H129 or YTA321 (anti-CD4), YTS169 (anti-CD8), MI/70 (anti-Mac-1), RB6-8C5 (anti-Gr-1), T3.24.1 (anti-Thy-1), IM781 (anti-CD44) and Ter119 (anti-erythroid marker), followed by flow cytometric analysis in a FACScan (BD Biosciences, San Jose, CA, USA). Staining was performed in the presence of anti-Fcγ receptor II (FcγRII) antibody (2.4G2) plus 2% normal rat serum to prevent non-specific binding of antibodies. Antibodies were produced in our laboratory and conjugated to biotin (Molecular Probes/Invitrogen, Carlsbad, CA, USA), fluorescein isothiocyanate (FITC, Molecular Probes), cyanine 5 (Cy5, Amersham/GE Healthcare, Sydney, Australia), R-phycoerythrin (R-PE, Prozyme), or allophycocyanin (APC, Prozyme) according to the manufacturers' instructions. Biotinylated antibodies were visualised by secondary staining using FITC-, PE- or Tricolor-streptavidin conjugates (Caltag/Invitrogen, Carlsbad, CA, USA). Dead cells were excluded by staining with propidium iodide (2 μg/ml). Cell sorting was performed using a MoFlo (Cytomation, Fort Collins, CO, USA) or DiVa (BD Biosciences) high-speed flow cytometer.

Enzyme-linked immunosorbent assay (ELISA). Immunoglobulin levels were measured using ELISA as described.[66] ELISA plates were coated with goat anti-total mouse immunoglobulin antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) in carbonate buffer. For measurement of total IgG levels, biotinylated goat antibodies specific to mouse IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associates) were combined for detection, followed by incubation with avidin-HRP and enzymatic detection. For determination of IgM levels, a biotinylated goat anti-IgM antibody was used as the secondary reagent.

Platelet clearance analysis. Mice were injected intravenously (i.v.) with 600 μg N-hydroxysuccinimidobiotin (NHS-biotin; Sigma-Aldrich, Sydney, Australia) in buffer containing 140 mM NaCl and 10% DMSO. At various time points, whole blood was isolated from the tail vein and mixed with Resuspension Buffer Mix, which comprised 25% (v/v) Aster Jandl anticoagulant (85 mM sodium citrate dihydrate, 69 mM citric acid anhydrans, 10 mM glucose, pH 4.6) and 75% (v/v) resuspension buffer (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$ hexahydrate, 0.5 mM $NaHCO_3$, 10 mM glucose, pH 7.4). The buffy coat, containing the platelet fraction, was separated by centrifugation at 125 g for 5 min and stained with FITC-conjugated rat anti-CD41 monoclonal antibody (BD Biosciences) and PE-conjugated streptavidin for 40 min at room temperature. Samples were washed in wash buffer (140 mM NaCl, 5 mM KCl, 12 mM sodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 6.0) by centrifugation at 860 g for 5 min. The platelet pellet was resuspended in resuspension buffer before flow cytometric analysis on a FACSCalibur (Becton Dickinson).

Adoptive transfer of platelets. Donor mice (wt or *bad*$^{-/-}$; $\sim$12 week-old) were injected with 0.1 μg/kg Alexa488-conjugated antibody specific for GPIβ subunit of GPIβ-V-IX for *in vivo* platelet labelling (Emfret). Labelling efficiency was assessed by flow cytometry, and only donor mice with $>$90% of the platelet population Alexa488$^+$ were used for experiments. Platelets were purified from these donors and injected intravenously into (untreated; $\sim$8 week-old) C57BL/6 recipients. At various time points, whole blood was isolated from the tail vein of recipients as described (see above under 'Platelet clearance analysis') and the disappearance of Alexa488$^+$ platelets analysed by flow cytometry.

Immunoblotting. Protein lysates were fractionated using 12% SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA), and electro-blotted onto nitrocellulose membranes (Hybond C-extra, Amersham/GE Healthcare). Membranes were probed with rabbit polyclonal anti-Bad (Cell Signalling, Danvers, MA, USA) or rat monoclonal anti-Bim (clone 3C5, Alexis/Enzo Life Sciences, Farmingdale, NY, USA) antibodies. Membranes were probed with mouse monoclonal anti-β-actin (clone AC-74, Sigma) antibodies to show equal loading. Horseradish peroxidase (HRP)-conjugated sheep anti-rabbit immunoglobulin (Ig), goat anti-rat Ig (Southern Biotechnology Associates) or sheep anti-mouse Ig antibodies (Chemicon/Millipore, Billerica, MA, USA) were used as secondary reagents and visualised using enhanced chemiluminescence (ECL) reagent (Amersham Biosciences/GE Healthcare). The molecular weight of proteins was measured using the rainbow molecular weight marker cocktail (RPN 756 Amersham Biosciences).

Cell culture and cell death assays. Cells were cultured in the high-glucose version of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 250 μM L-asparagine, 50 μM 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (JRH Biosciences, Kansas City, KA, USA). Activated B cells were

generated by culturing splenocytes for 3 days in medium supplemented with 20 μg/ml lipopolysaccharide (Difco) plus IL-2, IL-4 and IL-5 (each at 100 U/ml). Activated B cells were FACS sorted by staining with a cocktail of FITC-conjugated antibodies to CD4, CD8, Gr-1, Mac-1 and Ter119 plus PI and collecting the unstained ($FITC^-PI^-$) fraction. Sorted activated B cells were typically $>98\%$ pure as assessed by staining for the B lymphoid-specific marker B220. Activated T cells were generated by culturing spleen cells for 3 days in medium containing Concanavalin A (Con A, 2 μg/ml) plus IL-2 (100 U/ml), followed by 2 days of culture in medium containing IL-2 alone (100 U/ml). Viable T-cell blasts were FACS sorted by staining with a cocktail of FITC-conjugated antibodies to B220, CD19, Gr-1, Mac-1 and Ter119 plus PI and collecting the unstained ($FITC^-PI^-$) fraction. Sorted T-cell blasts were typically $>98\%$ pure as assessed by staining with antibodies to CD4 and CD8. Viability of cultured cells was assessed by staining with FITC-coupled Annexin V plus propidium iodide (2 μg/ml) and analysis using a FACScan (Becton Dickinson).

Generation of bone marrow chimaeric mice. Wild-type C57BL/6-Ly5.1, C57BL/6-Ly5.2 or *bad*$^{-/-}$ (C57BL/6-Ly5.2) mice were subjected to lethal γ-irradiation (2×5.5 Gy–3 h interval) from a ^{60}Co source at 3.6 Gy/min. Bone marrow cells (5×10^6) from C57BL/6-Ly5.2 or *bad*$^{-/-}$ (C57BL/6-Ly5.2) mice were used to reconstitute lethally irradiated C57BL/6-Ly5.1 mice. In addition, we reconstituted lethally irradiated *bad*$^{-/-}$ (C57BL/6-Ly5.2) mice with bone marrow from C57BL/6-Ly5.1 or *bad*$^{-/-}$ (C57BL-6-Ly5.2) mice to serve as controls. Animals were maintained on neomycin supplemented drinking water (1.6 g/l) for 14 days post-irradiation to prevent infection, and analysed 8–12 weeks post-reconstitution.

Whole body γ-irradiation and thymic lymphoma induction experiments. Mice were subjected to 2.5 or 5 Gy γ-irradiation. Bone marrow, spleen, thymus and lymph nodes were harvested 20 h post-irradiation. Single cell suspensions were prepared and total cell numbers determined by trypan blue staining and counting in a haemocytometer. Absolute numbers of cell subsets were calculated by multiplying the percentage of a cell type with the total organ cellularity. For studies on γ-irradiation-induced thymic lymphoma development, cohorts of wt, *bim*$^{-/-}$, *bad*$^{-/-}$, *bim*$^{-/-}$*bad*$^{-/-}$ and *p53*$^{-/-}$ mice aged between 25–35 days were subjected to 1.5 Gy γ-irradiation at 7-day intervals over 4 consecutive weeks as described.[44] Mice were monitored daily for signs of malignancy and sick animals killed and subjected to post mortem analysis.

Statistical analysis. Results are expressed as mean $\pm$ S.E. Statistical analysis was performed using the Student's *t*-test. *P*-values less than 0.05 were considered to be statistically significant differences. Kaplan–Meier tumour-free survival curves were constructed using GraphPad Prism software (Version 5), and statistical analysis was performed using the log rank (Mantel–Cox) test.

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)